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PRINCIPAL INVESTIGATOR: Sanford R. Simon, Ph.D.

CONTRACTING ORGANIZATION: State University of New York
at Stony Brook
Stony Brook, New York 11794-3366

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6. AUTHOR(S)
Sanford R. Simon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
State University of New York at Stony Brook
Stony Brook, New York 11794-3366

E-Mail: ssimon@notes.cc.sunysb.edu

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13. ABSTRACT *(Maximum 200 Words)*

We have evaluated novel nonantimicrobial chemically modified tetracyclines (CMTs) for management of invasive prostate cancer, based on preliminary results showing that one such CMT (CMT-3) inhibits matrix metalloproteinases (MMPs) as well as the serine proteinase human neutrophil elastase, reduces levels of released MMPs, and is selectively cytotoxic towards the human prostate tumor cell line LNCaP but not towards normal prostate stromal cells in culture. Because patients receiving CMT-3 in Phase I clinical trials at NCI have exhibited photoreactions, we screened all new CMTs with UV-A exposed NIH 3T3 cells to ensure that their phototoxicities *in vitro* are no greater than that of doxycycline. We evaluated the new CMTs for dose-dependent inhibition of collagenolytic and gelatinolytic activities of MMP-8 and MMP-9, as well as neutrophil elastase activity, and for differential cytotoxicity towards LNCaP cells, based on increased permeability to the dye SYTO 17 and failure to reduce the tetrazolium salt MTS. In addition to directly inhibiting proteolytic activity, the most promising CMTs downregulate expression of several markers of the invasive phenotype, including levels of the serine proteinase Prostate Specific Antigen released by LNCaP cells, MMP-9 released by PC-3 cells in response to TGF-β stimulation, and IGFBP-3 released by PC-3 in response to IGF-1 stimulation. These results suggest that the CMTs may be useful agents for management of prostate cancer because of their pleiotropic effects on proteinase activity, cell survival, and expression of multiple markers which correlate with invasiveness in tumors.

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A. Introduction

This final report outlines our progress on the tasks outlined in the Statement of Work for this project on the use of chemically modified tetracyclines (CMTs) in management of prostate cancer. Those tasks included: 1) screening normal prostate stromal and epithelial cells, prostate tumor cell lines, and prostate tumor explants for expression of proteinases; 2) measuring degradative activity of the prostate tumor cells towards stromal substrates; 3) evaluating the cytotoxic activity of chemically modified tetracyclines towards normal and tumor-derived prostate cells; 4) and 5) determining the capacity of the chemically modified tetracyclines to inhibit degradative activity of normal and tumor-derived prostate cells and the proteinases they may express; and 6) comparing new generation tetracycline derivatives to the then current lead candidate for development as drugs for use in management of prostate cancer. The progress we have made on the different tasks reflects the areas of greatest interest expressed by the USAMRMC in its critique of the original proposal. We have responded especially to the interest expressed by the Army in the development of new "second generation" chemically modified tetracyclines for use in the treatment of prostate cancer (Task 6). The three strategies for management of prostate cancer which we have attempted to address in this project through identification of potentially therapeutically useful chemically modified tetracyclines can be summarized as 1) inhibition of multiple sources of proteolytic activity associated with invasive tumors; 2) downregulation of the distinctive responses of tumors to soluble growth modulators as well as components of the interstitial stroma which support the expression of the invasive phenotype; and 3) use of agents which are cytotoxic selectively or preferentially to tumor cells and not to normal surrounding tissue.

The National Cancer Institute has continued to pursue clinical trials on the chemically modified tetracycline described in our original Statement of Work (6-demethyl-6-deoxy-4-de(dimethylamino)tetracycline or CMT-3). The criteria for admission of patients to these trials included failure on all other chemotherapies, and it was understood that the primary objective of the trials was to assess safety of the drug rather than efficacy. As a result of observations of patients participating in the trial at NCI, a significant incidence of dermatologic complications associated with phototoxicity of CMT-3 were noted. These dermatologic complications consisted primarily of sunburn-like lesions which were in all cases located on sites exposed to sunlight, including face, arms and hands. These reactions were not deemed so serious as to suspend further trials of CMT-3, which are now being undertaken at a consortium of sites across the country on patients with a variety of tumors other than primary prostate cancer or its metastases. Nevertheless, we responded to these results by accelerating our efforts to identify "new generation" CMTs which have significantly less phototoxicity than CMT-3 while retaining a profile of inhibition of matrix metalloproteinases as well as the serine proteinase human neutrophil elastase. Additionally, we have sought to identify CMTs which exhibit some selective cytotoxicity *in vitro* towards human tumor cells, especially established human prostate tumor cell lines, while displaying relatively low cytotoxicity towards normal human cells in culture. These studies have all been undertaken with the cooperation of CollaGenex Pharmaceuticals, Inc, which has provided the CMT-3 for the NCI clinical trials as well as all the new CMTs for our investigative work.

B. Progress on Tasks in Statement of Work

1. Phototoxicity Screening of New CMTs (Task 6)

In an effort to respond to the reported phototoxicity of CMT-3 in clinical trials, we have screened approximately thirty different tetracycline derivatives for phototoxicity to NIH 3T3 murine fibroblasts, using a standardized protocol in collaboration with colleagues at the Institute for In Vitro Sciences (IIVS) in Bethesda, MD. Our criteria for inclusion of new tetracycline derivatives for

further study were that they exhibit less phototoxicity than CMT-3 or than doxycycline or minocycline, tetracyclines which trigger an incidence of photoreactions in patients which is regarded as acceptable by the FDA. This phototoxicity screen involved incubation of the 3T3 cells for 50 minutes with the test compound in the dark or exposed to 5 Joules/cm^2 UV-A light, and provides some limited information about acute cytotoxicity as well as phototoxicity. Phototoxicity was measured as the IC_{50} for UV-exposed cells and was related to the cytotoxicity towards cells cultured in the dark to obtain a PhotoIrritancy Factor (PIF) or, for those compounds which displayed minimal cytotoxicity in the dark, was reported as a Mean PhotoEffect (MPE). As mentioned in the Introduction, we have arbitrarily disqualified CMTs for further consideration as potential drug candidates in this project if they showed greater phototoxicity in the *in vitro* assay than doxycycline. The two most phototoxic CMTs identified in this screen have been CMT-3 and CMT-8, both of which are much more phototoxic than doxycycline. However, several derivatives of these two CMTs have phototoxicities equivalent to or less than that of doxycycline. The results for several minimally phototoxic derivatives of CMT-3 are shown in Table 1. In addition to the listed derivatives of CMT-3, the 4-de(dimethylamino) derivative of minocycline, CMT-310 (formerly identified among the ten original chemically modified tetracyclines as CMT-10) was found to be particularly nonphototoxic, as were two of its derivatives, CMT-1001 and CMT-1002. A more detailed presentation of the methodology employed in this work by the IIVS team and by Elizabeth Roemer in our laboratory is included in the Appendix [1].

2. Antielastase Activity of New CMTs (Tasks 5 and 6)

Neutrophil elastase activity, which is normally associated with inflammatory cells, has been detected in especially invasive human breast tumors [2]; an elastase activity has also been reported in several Dunning rat prostate tumor cell lines, including MatLyLu [3]. We have used several types of measurements to assess antielastase activity of new CMTs. The most straightforward assay involves amidolysis of the chromogenic oligopeptide substrate, methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (MeOSAAPVPNA), by purified human neutrophil elastase. Using the purified enzyme and the oligopeptide substrate, we have shown that the CMTs are simple competitive inhibitors of elastase activity for which K_I values could be computed from analysis of Dixon plots. The inhibition of elastase activity reflects hydrophobic interactions between the enzyme and the CMTs. In the presence of serum albumin, the inhibitory potency of these hydrophobic CMTs is diminished as they bind to the hydrophobic pocket of the plasma protein. Human pancreatic elastase, which has a lower affinity than the neutrophil enzyme for a variety of hydrophobic anionic inhibitors, is much less sensitive than neutrophil elastase to inhibition by the CMTs. The CMTs are inactive as inhibitors of pancreatic trypsin or chymotrypsin. Furthermore, the trypsin-like serine proteinase known as TAT (Tumor Associated Trypsin) produced by a number of tumor cell lines, including COLO 205 human colon carcinoma cells [4], which we have shown to be capable of activating both MMP-2 and MMP-9 *in vitro*, is not sensitive to inhibition by CMTs. Representative results of antielastase K_I determination for a number of the new generation CMTs are summarized in Table 1. Some of these results have also been presented at the 1999 Annual Meeting of the Society for In Vitro Biology [5]; an abstract is included in the Appendix. Of the new CMTs, CMT-306 and CMT-308 have antielastase activity comparable or better than that of CMT-3, and, as we report below, these CMTs are good inhibitors of MMP-8-mediated collagenolysis and MMP-9-mediated gelatinolysis as well. In studies carried out in collaboration with Dr. L.M. Golub and his colleagues in the School of Dental Medicine at Stony Brook, we found that these two compounds reach serum concentrations in the rat comparable to those of doxycycline after administration of comparable oral doses, and have half lives in the circulation of $\geq$ 8 h. CMT-303 and CMT-315 also

inhibit all three enzymes, but CMT-303 shows less oral bioavailability in rats than doxycycline and CMT-315 appears to be converted into a metabolite of unknown nature after oral administration to rats.

A few of the more promising CMTs have also been checked for their capacity to inhibit the degradation of insoluble elastin by neutrophil elastase activity. Further confirmatory criteria for antiproteolytic activity of the new CMTs have been based on measurements of inhibition of degradation of a complete interstitial extracellular matrix (ECM) derived from R22 rat smooth muscle cells, using cystic fibrosis sputum or live human neutrophils as sources of proteolytic activity. We have previously shown that the degradation of this interstitial matrix by neutrophils or purulent secretions which are principally composed of neutrophil proteases is mediated primarily by neutrophil elastase rather than by matrix metalloproteases. The assays with elastin and R22 ECM have confirmed the apparent order of potency of the CMTs as inhibitors of neutrophil elastase as estimated from K_I determinations with oligopeptide substrates in solution. Some of these results have also been presented at the 1999 Annual Meeting of the Society for In Vitro Biology and are summarized in abstracts included in the Appendix [5,6].

3. New CMTs as Inhibitors of Matrix Metalloproteinases

a. Engineered Urokinase Assays (Tasks 5 and 6)

Matrix metalloproteinases (MMPs), whether produced by the tumor cells themselves or by the surrounding stroma, have been implicated in invasiveness of a number of tumors. We have employed several types of assays to screen potency of inhibition of the proteolytic activity of matrix metalloproteinases (MMPs) by CMTs. One assay makes use of a novel substrate for MMPs developed by Verheijen *et al*, [7] who replaced the normal plasmin-sensitive activation sequence in human prourokinase with an amino acid sequence which is cleaved not by serine proteinases but rather only by MMPs. MMP activity generates urokinase activity which is then detected by amidolysis of a chromogenic urokinase-specific oligopeptide p-nitroanilide. The advantage of this assay is that considerable amplification of the MMP proteolytic activity is achieved by using another enzyme as the substrate. In the commercially available form of this assay, the MMP to be assayed is first "captured" by an antibody linked to a 96-well microplate. If the MMP is already in its "active" state, its capacity to cleave the engineered urokinase may be assayed immediately. If "total" activity is desired, any latent proenzyme can be activated by an organomercurial (p-aminophenyl mercuric acetate, APMA) on the plate. After washing the excess reagent away, the activated MMP is incubated with the engineered prourokinase and the urokinase substrate in the presence or absence of a selected CMT in a kinetic microplate reader and the exponentially increasing rate of p-nitroaniline release is recorded for 4 hours. We have demonstrated that the CMTs do not inhibit the APMA-mediated activation of MMP-9 in this assay. Moreover, if the activated MMP-9 is then allowed to activate the engineered prourokinase prior to addition of the urokinase substrate and the CMTs, none of the tested CMTs inhibit the active urokinase generated in this fashion. Thus, in our hands, the CMTs we have tested specifically inhibit the proteolytic cleavage of an MMP-specific activation domain of the engineered prourokinase. These results, some of which were presented at the 1999 Annual Meeting of the Society for In Vitro Biology [8], are included in the data in Table 1. At this time, we have tested inhibition of MMP-9 proteolytic activity by a number of CMTs in this assay, but we are now evaluating inhibition of MMP-2 activity as well, using this assay as well as other assays described below, since its capacity to cleave the engineered urokinase is at least as great as that of MMP-9. These two gelatinolytic MMPs are produced in varying ratios by a number of prostate tumor cell lines, as can be seen in the attached Figures 1, 5, and 6.

b. Other Proteolytic Assays - Zymography, Collagenolysis, Gelatinolysis, ECM Degradation (Tasks 1, 2, 4, 5, and 6)

Using gelatin zymography, we have confirmed that MMP-2 and MMP-9 are both released by LNCaP prostate tumor cells [Figure 1]. PC-3 cells release low levels of MMP-9 when maintained in basal culture medium, but significantly upregulate their expression of MMP-9 in response to stimulation with TGF-β or IGF-1 [Figures 5, 6 and 8], as we recently reported at the 2000 meeting of the Society for Basic Urologic Research [9; an abstract is included in the Appendix]. Because many prostate tumors secrete and are stimulated by these growth factors in an autocrine fashion *in vivo*, it may be concluded that the elevated levels of MMP-9 released in response to these factors are likely to be of physiological relevance. The levels of MMP-2 secreted by the PC-3 cells are maintained at a constant constitutive level which is lower than the levels of MMP-9 secreted in the presence of growth factors; at this time we have not characterized levels of tumor cell membrane-associated MMP-2. We have shown that CMT-3 inhibits the gelatinolytic activity of both these MMPs when it is included in the incubation medium used to develop zymograms [10]. Moreover, when the supernatant medium from MMP-2-producing COLO 205 cells or the MMP-9-overproducing E-10 subclone of MDA-MB-231 cells was incubated with the R22 rat smooth muscle cell-derived ECM in our matrix degradation assay, both supernatants were capable of degrading the matrix after activation of the MMPs by tumor-associated trypsin (TAT) [10]. (TAT-mediated activation, which is similar to the plasmin-mediated activation of MMPs reported for the PC-3 prostate tumor line, takes place spontaneously during culture of the COLO 205 cells [18] but requires an *in vitro* incubation step for the E-10 culture supernatants, since these breast cancer cells do not synthesize their own TAT.) This ECM degradation could be inhibited by addition of 1,10-phenanthroline, implicating the MMPs in the supernatants as the source of the degradative activity. Moreover, ECM degradation by both TAT-activated supernatants in our assay could be inhibited by CMT-3 in a dose-dependent fashion [10,11]. We discuss the effects of the CMTs on expression of MMPs by prostate tumor cell lines below.

Our team has measured MMP-mediated collagenolysis using fluorescein-labeled type I collagen as a substrate. The resulting 1/4 length and 3/4 length products generated by the single MMP-catalyzed cleavage are much less stable at elevated temperatures than the native type I collagen triple helix and are further degraded to trichloroacetic acid-soluble fragments by a broad spectrum proteinase. The collagenolytic assay has the advantage of employing a substrate which is closer to the "natural" MMP substrates, but collagenolysis cannot be followed continuously. A variant of this assay employs gelatin which has been so heavily labeled with a fluorescein-like fluorescent probe that emission is quenched by Förster energy transfer. Gelatinolytic cleavages separate the individual probes and result in the appearance of a fluorescent signal. This new substrate allows us to measure gelatinolysis continuously with purified MMP-2 or MMP-9 or tumor cell supernatants as the sources of proteolytic activity. Recent results we have obtained for inhibition of MMPs by the new generation CMTs are reported in Table 1. Since the concentrations of the substrates used in the various assays for collagenolytic and gelatinolytic activity are very different, it is difficult to express the potencies of the different CMTs towards the MMPs as true K_I values. Using the engineered urokinase assay, we have determined true K_I values for CMT-3 and CMT-308 against MMP-9 of ~4 μM and ~1 μM respectively.

4. Effects of CMTs on Levels of Proteins Released by Normal and Tumor Cells (Tasks 1 and 6)

In the course of our studies on COLO 205 cells and the E-10 clone of MDA-MB-231 cells, we observed that CMT-3 not only inhibits MMP enzymatic activity but also reduces the levels of MMP-2 and MMP-9 in the supernatant medium of cultures which had been maintained for 48 hours in its presence [10,11]. As discussed in the following section, the mechanism for reduced levels of MMP-2 in cultures of COLO 205 cells maintained in the presence of CMT-3 may be due in part to cytotoxicity of this derivative on the colon carcinoma-derived cell line (LD_{50} = 10-20 μM), but diminution of the levels of MMP-9 released by the parental line of MDA-MB-231 cells as well as its overproducing E-10 subclone was achieved at the same 10 μM and 20 μM levels of CMT-3 which are not cytotoxic to this breast tumor cell line. We have since determined the effects of the different new CMTs on levels of gelatinolytic MMPs and the kallekrein-like serine proteinase Prostate Specific Antigen (PSA) produced by prostate tumor cell lines in culture.

Some of our first studies on prostate tumor cell lines were carried out with LNCaP cells. It has been suggested that this cell line is a model of early stage prostate cancer because it retains responsiveness to androgens, unlike the PC-3 and DU-145 lines, which are androgen-insensitive and are therefore considered to be more like late stage cancer [12]. LNCaP cells release a number of proteinases into the culture medium: we have already referred to our results in which MMP-2 and MMP-9 could be detected in LNCaP culture supernatants by gelatin zymography. When LNCaP cells are cultured in the presence of subcytotoxic levels of CMT-3, the levels of MMP-9 in the culture medium are diminished in gelatin zymograms, while the levels of MMP-2 are unaffected.

In addition to the MMPs, LNCaP cells release the serine proteinase PSA into the medium [13]. The role of this proteinase in invasiveness *in vivo* is still controversial, although some reports suggest that it is not merely a marker, but also a participant, in progression of the invasive phenotype in prostate cancer [14]. As the disease advances, especially after metastasis, PSA levels may then begin to decline, and in keeping with the view that PC-3 and DU-145 are models of advanced cancer, they do not release significant levels of PSA. We have screened several derivatives of CMT-3 for their effect on PSA levels released by LNCaP cells, using an ELISA which detects both free PSA and its complex with α_1-antichymotrypsin [9]. CMT-3 and CMT-306 are both quite cytotoxic to LNCaP cells, as discussed below, whereas CMT-308 displays much less cytotoxicity, even at high doses up to 100 μM. In the presence of 5 μM CMT-3 or 20 μM CMT-306, approximately 50% of the LNCaP cells maintained in complete, 4% serum-containing medium [15] were killed, as judged by assays of viable and total cell number using membrane-permeant and impermeant nucleic acid-binding fluorescent dyes [Figure 2]. However, the levels of PSA released by the remaining, viable cells were markedly diminished in the presence of these two CMTs: the PSA levels normalized per remaining viable cell were reduced by ~84% when the cells were cultured in the presence of 5 μM CMT-3 and by ~76% in the presence of 20 μM CMT-306 [Figure 3]. In the presence of concentrations of CMT-308 up to 20 μM, the levels of PSA released by LNCaP cells were unaffected when the cells were maintained in complete culture medium [Figure 3]. However, when the medium was "stripped" with charcoal and dextran and phenol red was excluded to remove all steroid-like activity, a significant decline in levels of PSA released by cells cultured in as little as 10 μM CMT-308 could be detected [Figure 4]. If the medium was supplemented with dihydrotestosterone (DHT), a hormone dose-dependent increase in PSA levels could be detected in the presence of CMT-308, although even in the presence of optimal DHT, PSA levels were diminished by ~30% when 100 μM CMT-308 was present in the culture medium. There was no significant cytotoxicity of CMT-308 up to 100 μM detected at any level of DHT [Figure 4].

TGF-β is a growth factor which is produced at elevated levels in bone, an especially favorable site for growth of prostate cancer metastases. The loss of the "normal" response to TGF-β, which is seen as growth inhibition in primary cultures of nontransformed epithelial cells, marks several prostate tumor cell lines. LNCaP cells are reported to lack TGF-β receptors, and in our gelatin zymograms, we saw no effect of TGF-β on MMP levels released by these cells in culture [Figure 1]. PC-3 cells, on the other hand, do respond to TGF-β. As shown in Figures 5 and 6, doses of TGF-β from 0.1 ng/ml to 10 ng/ml induced significant upregulation in levels of MMP-9 secreted into the medium, especially over the course of 48 hours, as detected on gelatin zymograms. This increased concentration of secreted MMP-9 was seen principally in the levels of aggregated forms of the MMP, but could be confirmed with an MMP-9 ELISA designed to detect all forms of the protein, including TIMP-complexes [Figure 7, front row of bars = 24 hour data, rear row = 48 hour data]. Addition of 20 μM CMT-308, which is completely noncytotoxic to PC-3 cells, had only a minimal effect on the levels of MMP-9 released by the cells maintained in the absence of TGF-β. However, 20 μM CMT-308 diminished the levels of MMP-9 released by PC-3 cells maintained in the presence of concentrations of TGF-β as high as 10 ng/ml down to levels seen in supernatants from the cells maintained in the absence of TGF-β [Figures 5, 6, and 7].

Another growth factor which has been implicated in enhancement of the invasive phenotype in PC-3 and DU-145 cells is IGF-1 [16]. PC-3 cells maintained for 48 hours in the presence of concentrations of IGF-1 from 0.5 μg/ml to 2 μg/ml released somewhat higher concentrations of MMP-9 into the medium as detected on gelatin zymograms [Figure 8]. We were surprised to find that 20 μM CMT-308 appeared to cause even greater release of MMP-9 (especially as seen in the levels of aggregated species on gelatin zymograms) by PC-3 cells maintained in the presence of IGF-1. This paradoxical upregulation of MMP expression in the presence of CMT-308 is, we believe, a reflection of the downregulation by the CMT of another protein released by PC-3 cells in response to IGF-1. As shown in the Western blot in Figure 9, in the presence, but not in the absence, of IGF-1, PC-3 cells released significant levels of a binding protein for this growth factor, IGFBP-3. It has been proposed that autocrine regulation of prostate tumor cell lines by IGF-1 is modulated by IGFBP-3: the soluble IGF-1-IGFBP-3 complex cannot bind to the IGF-1 receptor and therefore in the presence of the binding protein, the growth factor's stimulatory effect on the cells is diminished [16]. In the presence of 20 μM CMT-308, the levels of IGFBP-3 fell to below the limit of detection on the Western blot. Thus, IGFBP-3 represents yet another protein secreted by prostate tumor cells which is apparently downregulated by CMTs. In this case, however, because the binding protein prevents activation of the cells by IGF-1, its downregulation results in more, not less, stimulation and release of MMP-9. It should also be recognized that MMP-9 is reported to degrade IGFBP-3, but the concentrations of CMT-308 we have employed should have been sufficient to inhibit the enzymatic activity of the MMP-9 produced by the PC-3 cells. At this time we suspect that the effects of IGF-1 on MMP expression may be mediated via a pathway which is itself insensitive to CMTs, whereas the effects of TGF-β appear to be mediated via a CMT-sensitive pathway.

CMTs also affect levels of other proteins released by a variety of normal and tumor cells. We have shown that lipopolysaccharide-stimulated human monocytes and macrophages as well as the human monocytoid cell line Mono Mac 6 release lower levels of TNF-α after exposure to the CMTs, but this may reflect tetracycline inhibition of the metalloproteinase Tumor Necrosis Factor Converting Enzyme (TACE), a member of the ADAM family, rather than transcriptional regulation. However, the levels of IL-10, an anti-inflammatory cytokine, are decreased after incubation of monocytes with CMT-3 but upregulated after incubation with the noncytotoxic CMT-308. This result may have implications for regulation of survival and invasiveness of prostate tumors, as a

recent report indicates that IL-10 downregulates gelatinolytic MMP production and upregulates TIMP production in primary human prostate tumor cells in culture [17].

5. Effects of CMTs on Cell Survival (Task 3)

CMT-3 is especially cytotoxic towards the LNCaP cell line. This cytotoxicity, which is dose dependent, can be observed after exposure periods as short as a few hours. The PC-3 cell line is sensitive to CMT-3 as well, but cytotoxicity is not apparent until 48-72 hours have elapsed. A third prostate cancer cell line, DU-145, is quite resistant to all but high doses of CMT-3 even after 72 hours, while normal human prostate stromal cells are also highly resistant [10 and Figures 10-13]. We have demonstrated that CMT-3 also shows variable cytotoxicity towards other cancer cell lines: COLO-205 cells are quite sensitive to this derivative, with an IC_{50} on the order of 10-20 μM after 48 hours of exposure, whereas MDA-MB-231 cells as well as their MMP-9-overexpressing E-10 transfected clone, and Mono Mac 6 cells, which also express MMP-9, are resistant to 50 μM CMT-3. Criteria for cytotoxicity we have employed have included reduction of the tetrazolium dye MTS to its formazan, uptake of plasma membrane-impermeant DNA-binding dyes such as SYTO 17, and release of the cytosolic enzyme lactic dehydrogenase into the supernatant.

We have undertaken a comprehensive study of the effects of the CMTs on the capacity of the LNCaP cell line to exclude SYTO-17 from the nucleus. The cells were maintained in 48 well microplates on which the R22 interstitial ECM had been previously deposited. Cells were maintained in the presence of the CMTs for 24 or 48 hours prior to incubation with the SYTO 17 dye. Table 2 summarizes the apparent IC_{50} values for the different CMTs after 24 and 48 hours of exposure, based on SYTO 17 permeability. These results were also presented at the 1999 and 2000 Annual Meetings of the Society for In Vitro Biology [18,19]; abstracts are included in the Appendix. The results are consistent with our earlier observations that CMT-3 can begin to induce cytotoxicity within hours, while other tetracycline derivatives, such as doxycycline, are cytotoxic only after 48 hours of incubation.

The progressive loss of membrane integrity which renders cells permeable to SYTO 17 can be correlated with a progressive loss of mitochondrial reductase activity, detected as a failure to convert MTS to its formazan. With few exceptions, the different assays for cytotoxicity are in good agreement, permitting us to rank the new CMTs in order of their cytotoxic activity towards LNCaP cells. In the course of undertaking the MTS measurements, we performed assays on cells maintained in RPMI medium containing phenol red and 4% fetal bovine serum as well as phenol red-free medium containing dextran- and charcoal-stripped serum in order to address concerns that the combination of serum and indicator dye might be exerting some hormone-like effects on the cells in culture. Indeed, we have found that cells maintained in the presence of phenol red and whole serum display consistently greater sensitivity to the cytotoxic CMTs than cells cultured in phenol red-free medium with charcoal/dextran-stripped serum. Observations of morphologic changes indicate that cytotoxicity is associated with a distinctive change in properties of the cells in culture: rather than maintaining a confluent layer of adherent cells, the LNCaP cells begin to round up, detach, and form clusters in the presence of the more cytotoxic CMTs, whereas the cells retain their shape and adherence in the presence of the noncytotoxic CMTs. Table 2 includes data for the apparent IC_{50} values for the different CMTs after 48 hours of incubation with LNCaP cells in the presence and absence of phenol red, based on MTS assay results, along with descriptions of the morphologic changes which could be seen by light microscopy. It should be noted that changes in cell morphology including rounding, detachment, and loss of confluence in other cell types, such as endothelial cells, are typically associated with loss of invasiveness.

To follow up on our initial observations on the selective cytotoxicity of CMT-3 towards

prostate tumor cells but not towards normal prostate stromal cells, we have surveyed the effects of the newer CMTs on the R22 rat smooth muscle cells we routinely employ for elaboration of a stromal ECM [19], as well as both stromal and epithelial cells (not transformed) obtained from normal human prostate. The results show that only a very limited number of CMTs, including CMT-301, -302, and -303, but not CMT-3, show measurable cytotoxicity at doses of less than 50 μM towards the normal rat and human stromal cells after one day of incubation, as evidenced by failure to reduce MTS. CMT-3 and CMT-306 do begin to display some cytotoxicity after more extended exposure. However, among those CMTs which do not cause outright loss of reductase activity in the MTS assay, several (including CMT-3) cause morphologic changes such as rounding and loss of confluence at higher but still subcytotoxic doses [19]. We have limited data for the sensitivity of human epidermal keratinocytes to the CMTs and also data for normal human prostate epithelial cells: these initial measurements suggest that the epithelial cells are more sensitive than the stromal cells we have examined, although the apparent IC_{50} values for the human keratinocytes and prostate epithelial cells are consistently higher than the corresponding values for LNCaP cells cultured under the same conditions. The results for cytotoxicity of CMTs towards LNCaP, COLO 205, R22 rat stromal cells, and normal human epithelial keratinocytes are all summarized in Table 2; The cytotoxicity of some derivatives of CMT-3 towards normal human prostate epithelial and stromal cells is illustrated in Figures 14 and 15.

6. Other Normal and Tumor-Derived Prostate Cell Culture Studies (Tasks 4 and 5)

The typical experience of previous investigators is that "...lines are difficult to establish from explants of prostate tissue" [20]. Elizabeth Roemer in our team has carried out three explant cultures of freshly isolated prostate tumor tissue obtained from surgical resections performed by Dr. S. Ali Khan. After a period of 4 to 9 days, outgrowth of cells can be observed from about 5-10% of the tissue fragments. While the morphology of these cells is consistent with that of tumor rather than fibroblasts from the surrounding stroma, Ms. Roemer has not been able to carry the cells beyond a fourth passage. The cells do not appear to survive plating in 96 well plates, so we will have to design any future studies with explant cultures carefully to obtain maximum information from the limited material available.

We have also progressed in culturing normal human prostate epithelial cells for cytotoxicity testing with CMTs, as illustrated in Figure 14. These cells, from Clonetics Corporation, are maintained in a specialized medium prepared by the vendor. Normal human prostate stromal cells have also been provided by Clonetics Corporation, along with a special serum-free medium. Our studies using sequential enzyme digestion of the matrix produced by the prostate stromal cells show that this stromal ECM is considerably richer in trypsin-sensitive components and poorer in collagen than the R22 rat smooth muscle cell-derived ECM [21]; an abstract of this work is included in the Appendix. Projected modifications proposed by Clonetics for new formulations of their stromal cell culture medium may result in enhanced collagen synthesis by the confluent cells. The normal prostate stromal cells have also been used for evaluation of cytotoxicity of the different CMTs [Figure 15], using Clonetics' standard fibroblast medium for maintenance of the cells in culture. This medium is not suitable for supporting ECM synthesis, but does support maintenance of stromal cell viability in the absence of the CMTs.

C. Key Research Accomplishments

- Screened new CMTs for phototoxicity *in vitro* and identified several new compounds with phototoxicity no greater than that of doxycycline and less than that of CMT-300.
- Screened new CMTs for inhibition of human neutrophil elastase activity and for inhibition

of collagenolytic and gelatinolytic activities of MMP-8 and MMP-9 respectively and identified compounds with inhibitory potency comparable to that of CMT-300.

- Screened new CMTs for cytotoxicity towards tumor cell lines and normal cells in culture and identified several new compounds with selective or preferential cytotoxicity towards at least one prostate tumor cell line (LNCaP).
- Developed procedures for culturing explants of resected human prostate tumors and achieved outgrowth of cells from the explants. These efforts are in early stages but are proceeding well.
- Progressed on developing culture conditions for normal human prostate epithelial and stromal cells for use as targets in cytotoxicity assays and for synthesis of interstitial ECM. These efforts have been slowed by limited availability of appropriate culture media.
- Demonstrated apparent diminution of MMP-9 and PSA release by LNCaP cells in the presence of subcytotoxic levels of CMT-3 and CMT-306, indicating effects of CMTs on protein levels in addition to inhibition of enzyme activity.
- Demonstrated apparent androgen-sensitive diminution of PSA release by LNCaP cells in the presence of the noncytotoxic CMT, CMT-308.
- Demonstrated a dose- and time-dependent enhanced release of MMP-9 by PC-3 cells stimulated with TGF-β, and a diminution of the TGF-β-enhanced MMP-9 release by CMT-308 back down to the levels seen in unstimulated cells, further substantiating hypothesis that CMTs modulate levels of protein markers of the invasive phenotype.
- Demonstrated release of IGFBP-3 by PC-3 cells stimulated with IGF-1, but not by unstimulated cells, and demonstrated diminution of IGFBP-3 release to below detectible levels in the presence of IGF-1 and CMT-308, further substantiating the conclusion that CMTs affect levels of protein markers of invasiveness other than MMPs.

D. Reportable Outcomes

The most tangible outcome of this project is projected to be the identification of one or more of the most promising new CMTs for inclusion in the drug development program of CollaGenex Pharmaceuticals and for evaluation in clinical trials. At this time, we believe it is premature for us to identify any one of the new CMTs as so unquestionably superior to the others as to merit such commitment of resources. However, several of the new CMTs appear to overcome the primary complication of undesirable photoreactions encountered in clinical trials with CMT-300, so that significant progress towards this outcome should be achieved within the time frame of the project.

More immediate outcomes are reflected in presentations at the 1999 and 2000 Annual Meetings of the Society for In Vitro Biology and the 2000 Fall Meeting of the Society for Basic Urologic Research. These presentations took the form of posters as well as an oral presentation. These presentations are listed below (abstracts are included in the Appendix):

1. Simon, S.R., E.J.Roemer, W.Bellucci, Y.Gu, Q.L.Ying, C.Mannino, and E.Spero. (1999) Novel inhibitors in inflammation and Metastasis. *In Vitro* Cell. and Devel. Biol. 35:2A.
2. Bellucci, W.A., E.J. Roemer, S.R. Simon. (1999) A new screening assay for the evaluation of inhibitors of matrix metalloproteinases. *In Vitro* Cell. and Devel. Biol. 35:29A.
3. Boumakis, S., S.R.Simon and E.J.Roemer. (1999) Evaluation of the cytotoxicity of chemically modified tetracyclines (CMTs) on LNCaP prostate tumor cells. *In Vitro* Cell. and Devel. Biol. 35:31A.
4. Musacchia, L.C., Y.Gu, E.J.Roemer and S.R.Simon. (1999) Effects of chemically modified

tetracyclines (CMTs) on colon and breast cancer cells *in vitro*. *In Vitro* Cell. and Devel. Biol. 35:31A.

5. Sehgal,B., S.R.Simon and E.J.Roemer. (1999) Chemically modified tetracycline 300 and its derivatives inhibit extracellular matrix degradation by polymorphonuclear leukocytes and Cystic Fibrosis sputum. *In Vitro* Cell. and Devel. Biol. 35:31A.
6. Spero, E.F., E.J.Roemer, S.R.Simon and C.Ren. (1999) The effect of chemically modified tetracyclines on the inhibition of sputum elastase in the presence of polyanions. *In Vitro* Cell. and Devel. Biol. 35:31A.
7. Bellucci, W.J., E.J.Roemer, C.L.Ren, and S.R.Simon (2000) Role of matrix metalloproteinases in alpha-1-proteinase inhibitor degradation by neutrophil-derived proteases. *In Vitro* Cell. and Devel. Biol. 36:76A.
8. Scotto-Lavino, E., H.A.Sawka, S.R.Simon, and E.J.Roemer (2000) Evaluation of the composition of the extracellular matrix synthetized by human prostate stromal cells in culture. *In Vitro* Cell. and Devel. Biol. 36:76A.
9. Guilfoy, K., S.R.Simon, and E.J.Roemer (2000) Cytotoxic effects of chemically modified tetracyclines on R22 cells. *In Vitro* Cell. and Devel. Biol. 36:79A.
10. Kothari, M., and S.R.Simon (2000) Effects of chemically modified tetracyclines on matrix metalloproteinase and PSA in prostate tumor cell lines. Proc. Soc. Basic Urol. Res. - Fall Meeting:Friday 10/10/00.

Two publications have also resulted from this project:

11. Zerler, B, E.Roemer, H.Raabe, A.Sizemore, A.Reeves, and J.Harbell (2000) Evaluation of the phototoxic potential of chemically modified tetracyclines with the 3T3 neutral red uptake phototoxicity test. *Progress in the Reduction, Refinement, and Replacement of Animal Experimentation*, M. Balls, A-M. van Zeller, and M.E. Halder, eds. Elsevier. pp. 545-554.
12. Gu, Y., H.M.Lee, E.J. Roemer, L.Musacchia, L.M.Golub, and S.R.Simon (2001) Inhibition of tumor cell invasiveness by chemically modified tetracyclines. Curr. Med. Chem. 8:261-270.

E. Conclusions and Implications for Broader Project Goals

We may summarize our progress on the work outlined in our original proposal as follows: 1. We have evaluated a number of new CMTs provided by CollaGenex Pharmaceuticals, Inc. as potential "leads" for use in management of prostate cancer. Prior to these studies, no information regarding the biological activities of these new CMTs was available. We have based our evaluations on the criteria of significant antiproteinase activity towards MMPs and the serine proteinase neutrophil elastase, selective cytotoxicity towards at least one prostate tumor cell line but not towards normal cells at comparable doses, and low phototoxicity following UV-A exposure. On the basis of these criteria, as well as oral bioavailability studies in rats and solublity studies, we have identified six new CMTs, four of which are derivatives of CMT-300 (formerly CMT-3) and two of which are derivatives of CMT-310 (formerly CMT-10). The relevant properties of the new derivatives of CMT-3 are summarized in Tables 1 and 2. None of these compounds has less oral availability than the antimicrobial agents minocycline, doxycycline, or tetracycline, and none precipitates out of culture medium at doses which produce the desired biological responses. The development and implementation of criteria for selection of these derivatives for further study has

also been summarized in a presentation at the 1999 Annual Meeting of the Society for In Vitro Biology [11], and abstract of which is included in the Appendix.

2. We have obtained evidence that the new CMTs have general properties which identify them as potential anti-metastatic drugs even beyond their specific use for management of prostate cancer. To date, these properties include effects on the shape and growth characteristics of cells which may compromise their invasive potential, and the capacity to downregulate levels of matrix metalloproteinases, one serine proteinase, and one growth factor binding protein released by the cells into their surrounding environment. These additional properties further complement the selective cytotoxicity and antiproteolytic activity which we had originally observed for CMT-3 and which prompted us to propose its use in management of prostate cancer.

3. On the basis of results of clinical trials using CMT-3, we have had some concern that phototoxicity associated with this compound may be sufficiently problematic to restrict its future use to relatively acute or short term (< 2 weeks) applications. Accordingly, we have ensured that the new CMTs we have continued to investigate are all no more phototoxic than doxycycline, a tetracycline which has been administered on a long term basis to patients with acceptable levels of adverse side effects.

4. At this time, we have achieved outgrowth of tumor cells from a limited number of resected prostate cancers, and we have maintained normal human prostate stromal and epithelial cells in culture. We have also succeeded in preparing limited quantities of interstitial ECM from normal prostate stromal cells. We have evaluated cytotoxicity of the new CMTs towards normal prostate stromal cells and, to a more limited extent, towards normal prostate epithelial cells.

5. The evaluative criteria we have applied to characterize the new CMTs are intended specifically to identify a number of compounds for further consideration as candidates in the drug development program being undertaken by CollaGenex Pharmaceuticals for treatment of cancer. By concentrating our efforts on compounds which we have shown to possess the combined properties of low photoxicity, good antiproteinase activity, and selective cytotoxicity, we believe that the objectives of this project have been addressed.

Publications

1999 Simon, S.R., E.J.Roemer,W.Bellucci, Y.Gu, Q.L.Ying, C.Mannino, and E.Spero. Novel inhibitors in inflammation and Metastasis. *In Vitro* Cell. and Devel. Biol. 35:2A.

1999 Bellucci, W.A., E.J. Roemer, S.R. Simon. A new screening assay for the evaluation of inhibitors of matrix metalloproteinases. *In Vitro* Cell. and Devel. Biol. 35:29A.

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2000 Scotto-Lavino, E., H.A.Sawka, S.R.Simon, and E.J.Roemer. Evaluation of the composition of the extracellular matrix synthetized by human prostate stromal cells in culture. *In Vitro* Cell. and Devel. Biol. 36:76A.

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2000 Kothari, M., and S.R.Simon. Effects of chemically modified tetracyclines on matrix metalloproteinase and PSA in prostate tumor cell lines. Proc. Soc. Basic Urol. Res. - Fall Meeting:Friday 10/10/00.

2000 Zerler, B, E.Roemer, H.Raabe, A.Sizemore, A.Reeves, and J.Harbell. Evaluation of the phototoxic potential of chemically modified tetracyclines with the 3T3 neutral red uptake phototoxicity test. *Progress in the Reduction, Refinement, and Replacement of Animal Experimentation*, M. Balls, A-M. van Zeller, and M.E. Halder, eds. Elsevier. pp. 545-554.

2001 Gu, Y., H.M.Lee, E.J. Roemer, L.Musacchia, L.M.Golub, and S.R.Simon Inhibition of tumor cell invasiveness by chemically modified tetracyclines. Curr. Med. Chem. 8:261-270.

Personnel

Sanford R. Simon, Ph.D. - Principal Investigator
S. Ali Khan, M.D. - Collaborating Investigator
Elizabeth J. Roemer, M.A. - Senior Research Scientist (Simon laboratory)
Jackie Lie, B.A. - Research Assistant (assistant to Dr. Khan)
Wayne Bellucci, B.A. - Research Assistant (Simon laboratory)
Ying Gu, M.A. - Graduate Research Assistant (Simon laboratory)
Mansi Kothari, M.A. - Graduate Research Assistant (Simon laboratory)

note:
Lorne M. Golub, D.D.S. (Collaborating Investigator) also participated in this project but received no direct support; also several undergraduate students, including Stavroula Boumakis, Kimberly Guilfoy, Leo Musacchia, Elizabeth Scotto-Lavino, and Bantoo Seghal, participated in this project but received no direct support.

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[1] Zerler, B, E.Roemer, H.Raabe, A.Sizemore, A.Reeves, and J.Harbell. (2000) Evaluation of the phototoxic potential of chemically modified tetracyclines with the 3T3 neutral red uptake phototoxicity test. *Progress in the Reduction, Refinement, and Replacement of Animal Experimentation*, M. Balls, A-M. van Zeller, and M.E. Halder, eds. Elsevier. pp. 545-554.

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Table 1 - IC_{50} and KI (μM) of CMTs - MMP-8, MMP-9/-2, HNE Inhibition, and Phototoxicity

CMTs	IC_{50} μM MMP-8	IC_{50} μM MMP-9	IC_{50} μM MMP-2	K_I HNE μM	MPE / PIF
CMT-3	4	< 1 (K_I = 4)	1.4	44	0.92 / 820
CMT-302				11	0.41 / 46
CMT-303	4	8.5		10	0.53 / 15.5
CMT-305				104	0.78 / 17.7
CMT-306	7	4.9		77	0.032/1.06
CMT-307	1	5.2		64	0.017/1.32
CMT-308	1.5	4.2 (K_I = 1)		49	0.082/ 1.0
CMT-315	10			1.71	0.35 / 20.0

Table 2 - LD_{50}-μM of CMTs for Prostate and Colon Cancer and Normal Stromal and Epithelial Cell Lines

CMTs	LNCaP 1 day	LNCaP 2 day	LNCaP 2day +SH	LNCaP 2 day -SH	COLO 205	R22 nonprolif	R22 prolif	NHEK
CMT-3	7.5	6.9	9	28	41.5	nt >75%R	nt >75%R	13.96
CMT-301	25	15	8	18		8.6 >75%R	32.8 >75%R	
CMT-302	34	23.5	7	20		43.5 >75%R	35 >75%R	
CMT-303	30	21	10	45	36	52 >75%R	53 >75%R	6.5-14
CMT-306	33	25.5	18	42	nt	nt 0%R	nt 0%R	9
CMT-307			nt	nt		nt	nt	
CMT-308	nt	nt	nt	nt	nt	nt 0%R	nt 0%R	64
CMT-315	4	5	nt	nt	nt	nt	nt	100

+SH = contains androgen activity; -SH = androgen depleted. nt = nontoxic; %R = percent rounded at 20 uM

Effect of TGF beta on LNCaP cells

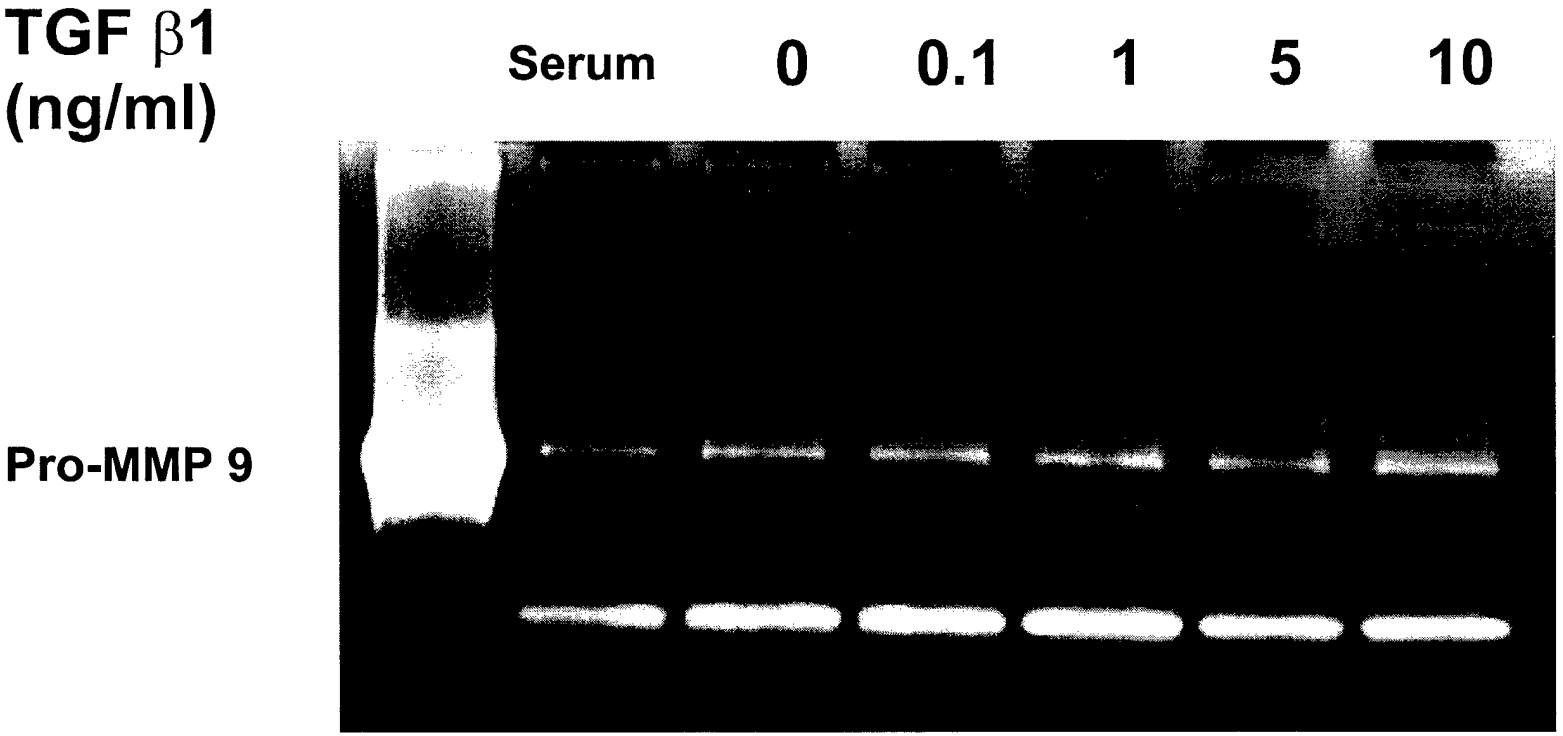


Figure 1

Cytotoxicity on LNCaP cells
Cell Number
120000
100000
80000
60000
40000
20000
0
LNCaP (control)
EtOH (0.5%)
CMT 300 (5uM)
CMT 306 (20uM)
CMT 308 (20uM)

Figure 2

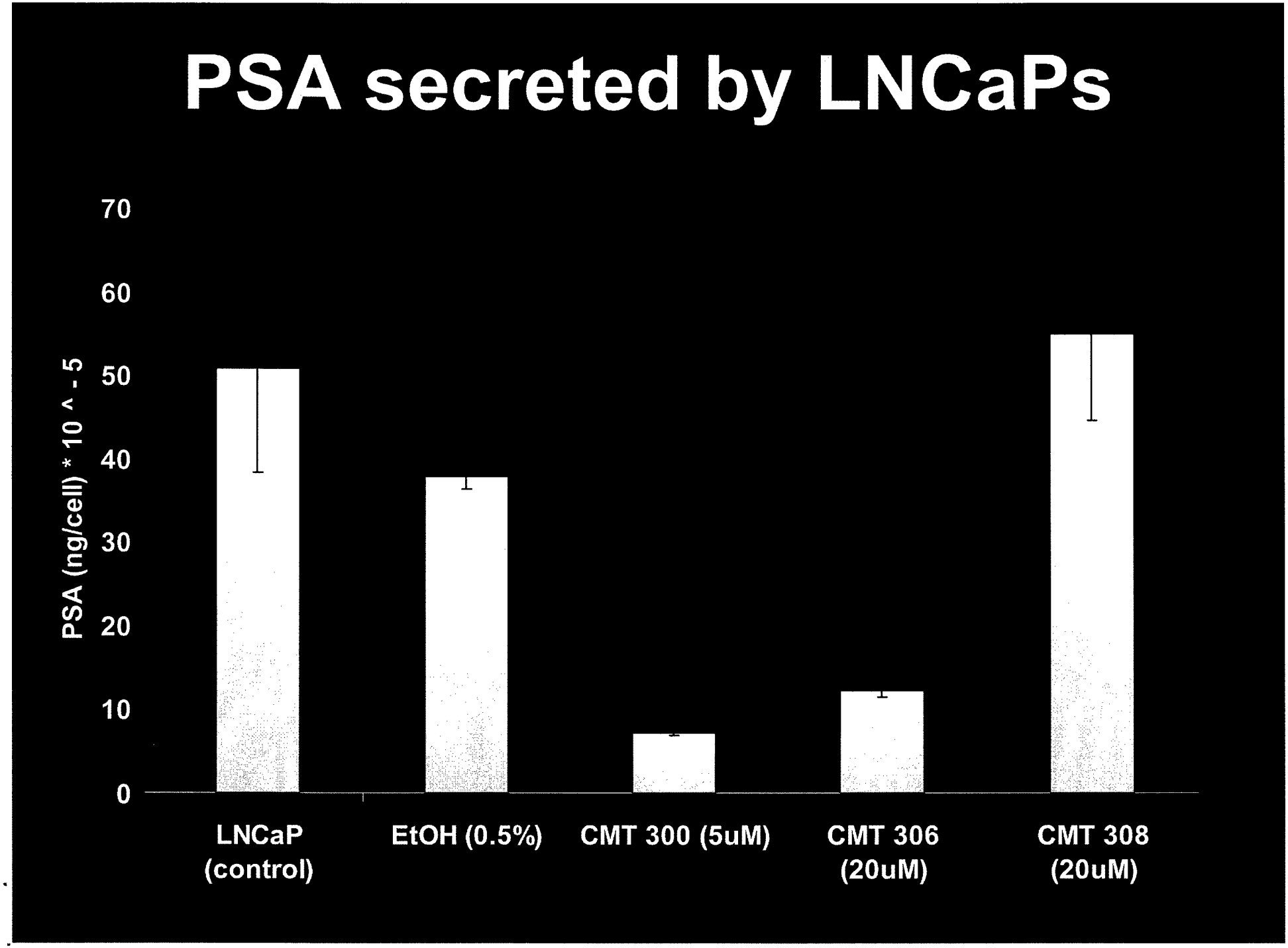

PSA secreted by LNCaPs
PSA (ng/cell) * 10 ^ - 5
70
60
50
40
30
20
10
0
LNCaP (control)
EtOH (0.5%)
CMT 300 (5uM)
CMT 306 (20uM)
CMT 308 (20uM)

Figure 3

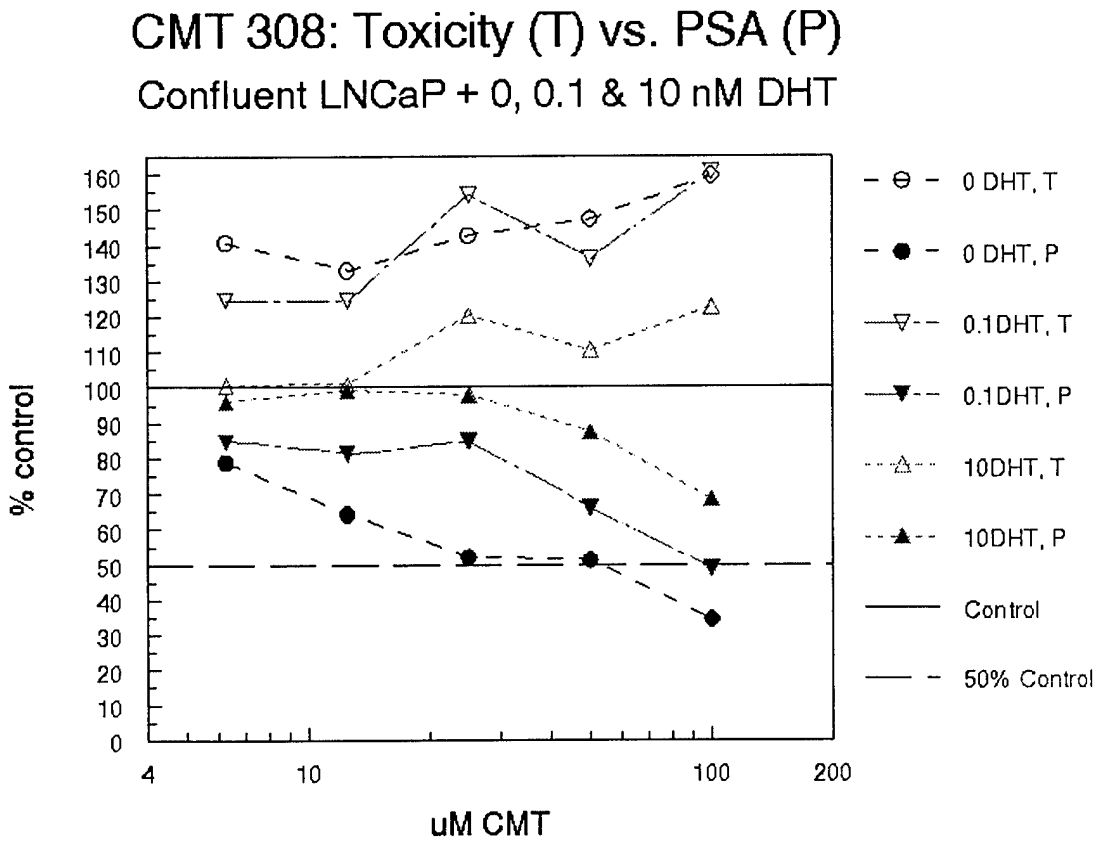

CMT 308: Toxicity (T) vs. PSA (P)
Confluent LNCaP + 0, 0.1 & 10 nM DHT
% control
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
4
10
100
200
uM CMT
0 DHT, T
0 DHT, P
0.1DHT, T
0.1DHT, P
10DHT, T
10DHT, P
Control
50% Control

Figure 4

Effect of TGF beta on PC-3 cells - 24 hrs

TGF β1 (ng/ml)	-	-	0.1	0.1	1	1	10	10
CMT 308 (20 uM)	-	+	-	+	-	+	-	+

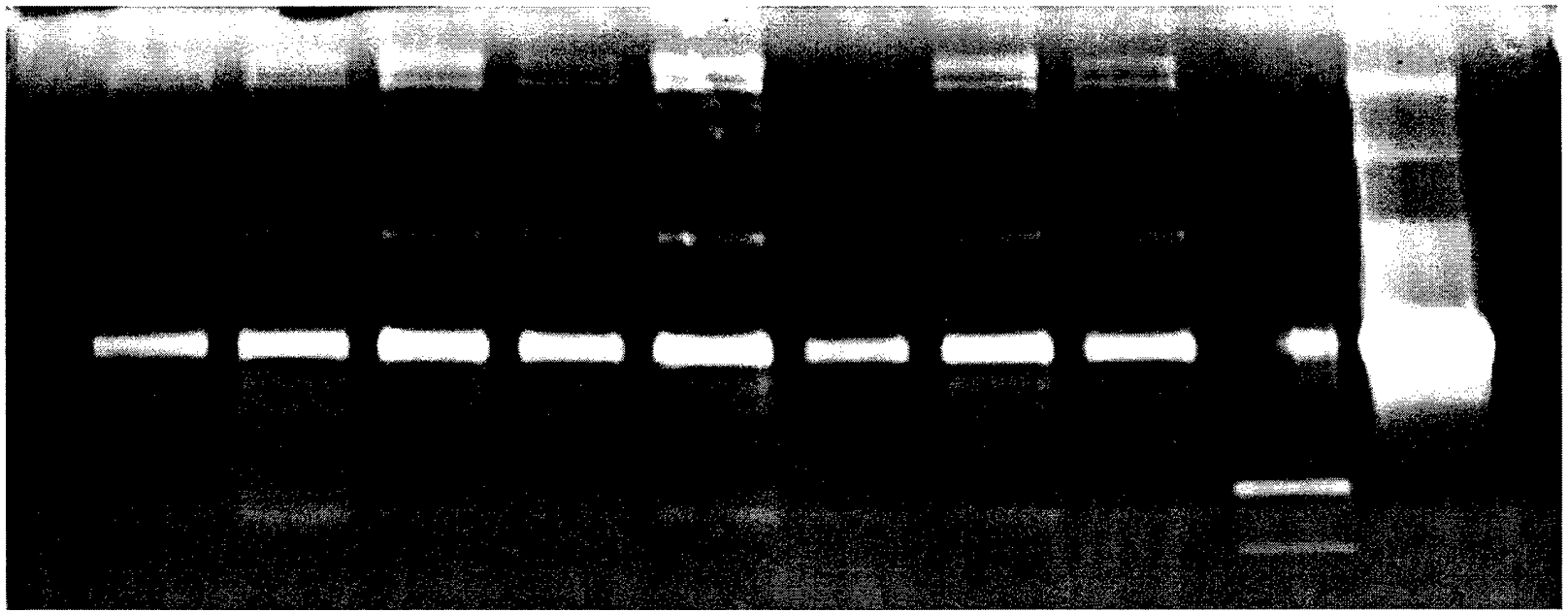


Figure 5

Effect of TGF beta on PC-3 cells - 48 hrs

TGF β1 (ng/ml)	-	-	0.1	0.1	1	1	10	10
CMT 300 (5 uM)	-	+	-	+	-	+	-	+

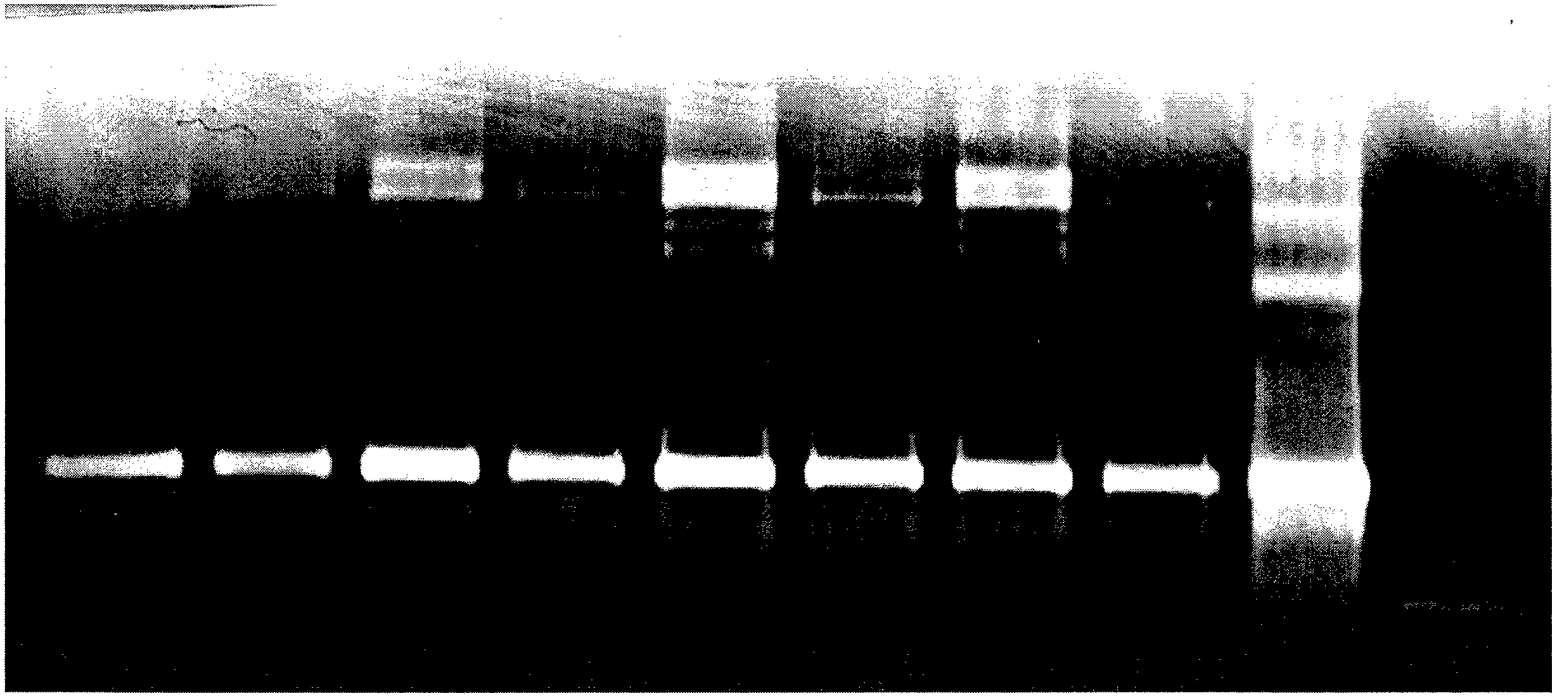


Figure 6

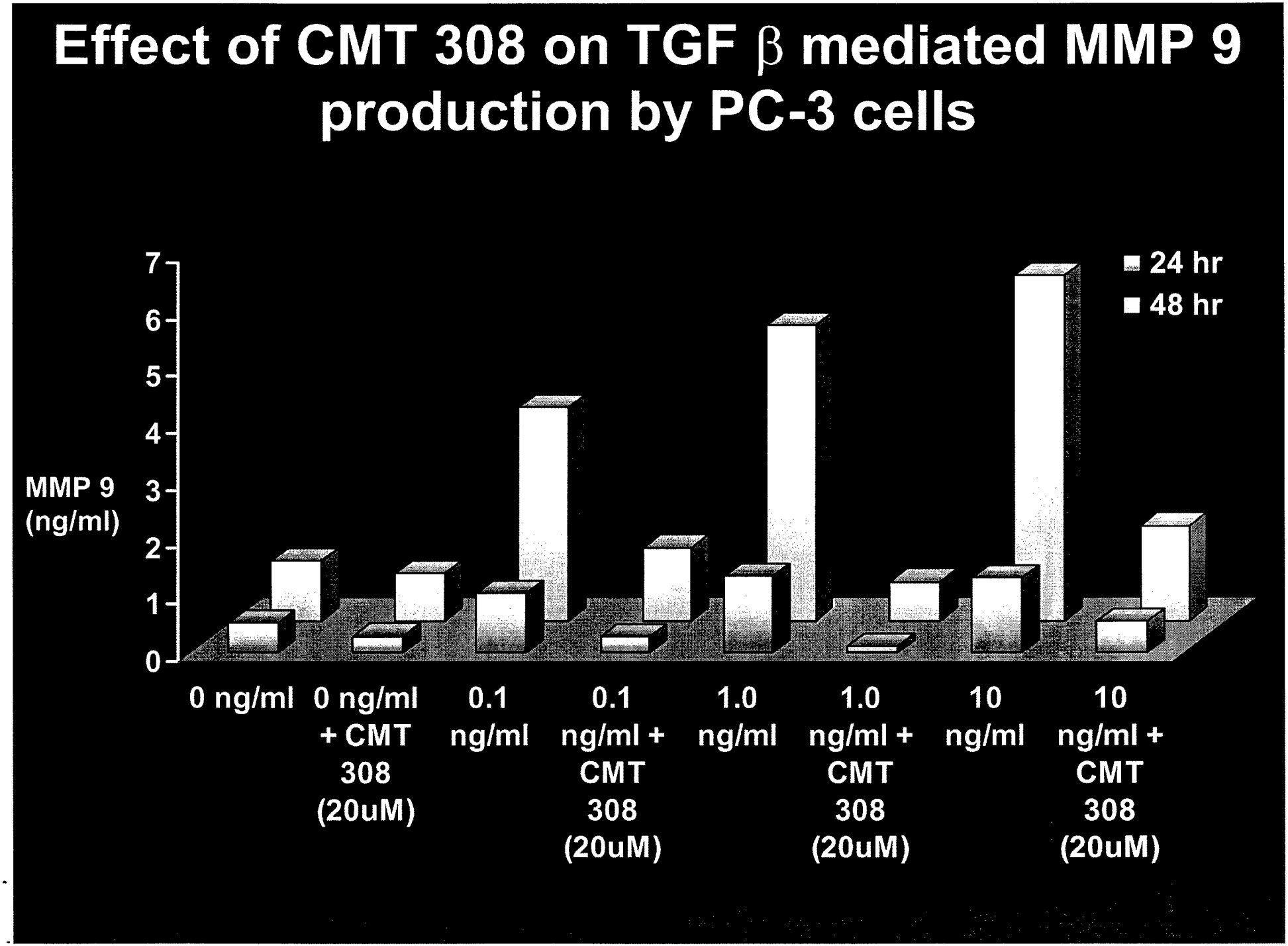

Effect of CMT 308 on TGF β mediated MMP 9 production by PC-3 cells
MMP 9 (ng/ml)
0
1
2
3
4
5
6
7
24 hr
48 hr
0 ng/ml
0 ng/ml + CMT 308 (20uM)
0.1 ng/ml
0.1 ng/ml + CMT 308 (20uM)
1.0 ng/ml
1.0 ng/ml + CMT 308 (20uM)
10 ng/ml
10 ng/ml + CMT 308 (20uM)

Figure 7

IGF-1 + 308 48Hr on PC-3 cells

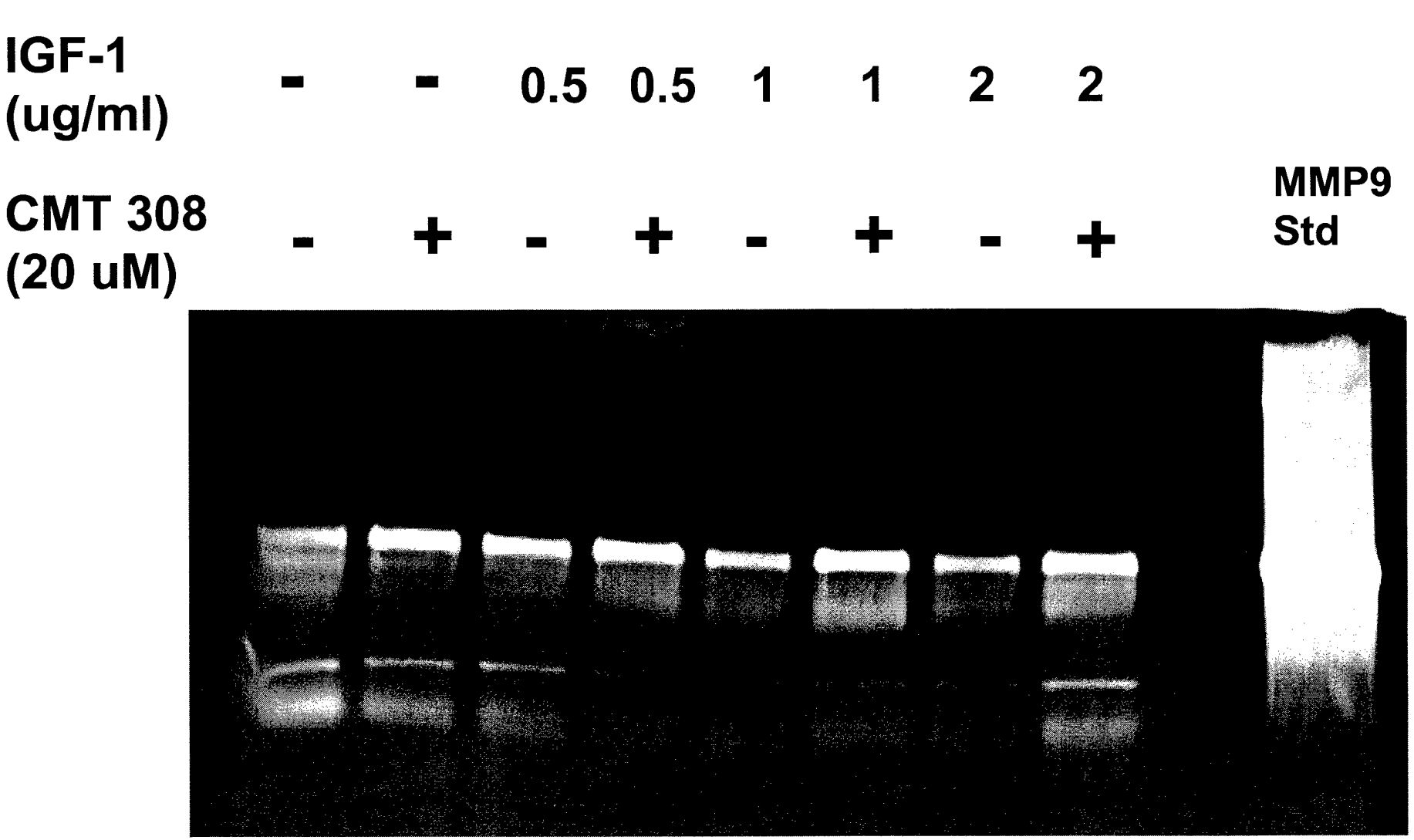


Figure 8

IGFBP-3 W. Blot B-13.0.5

IGF-1 (ug/ml)	-	-	0.5	0.5	1	1	2	2
CMT 308 (20 uM)	-	+	-	+	-	+	-	+

Figure 9

Figure 10

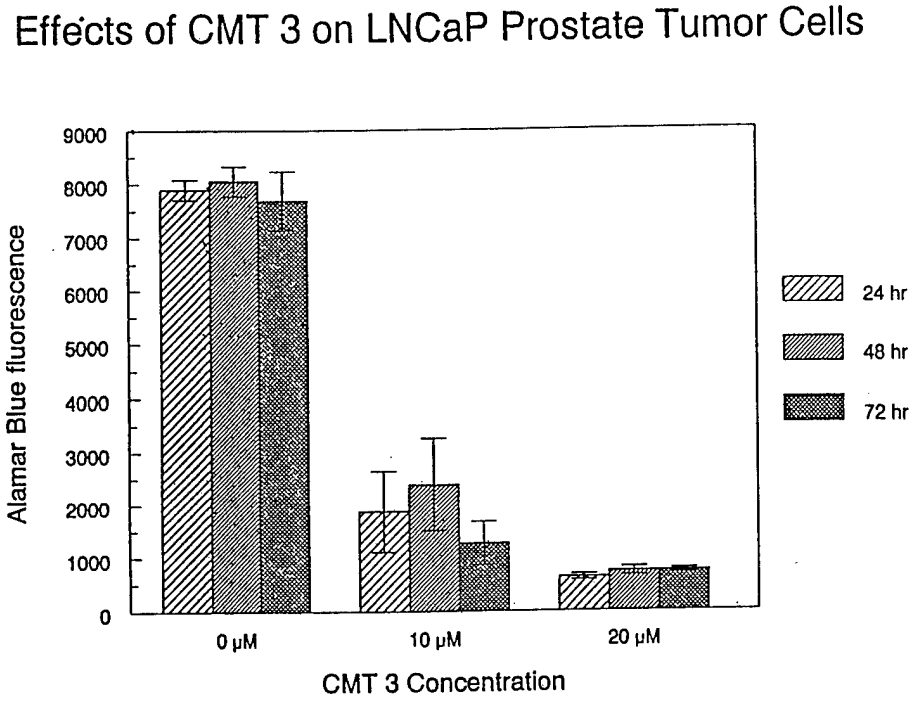


Figure 11

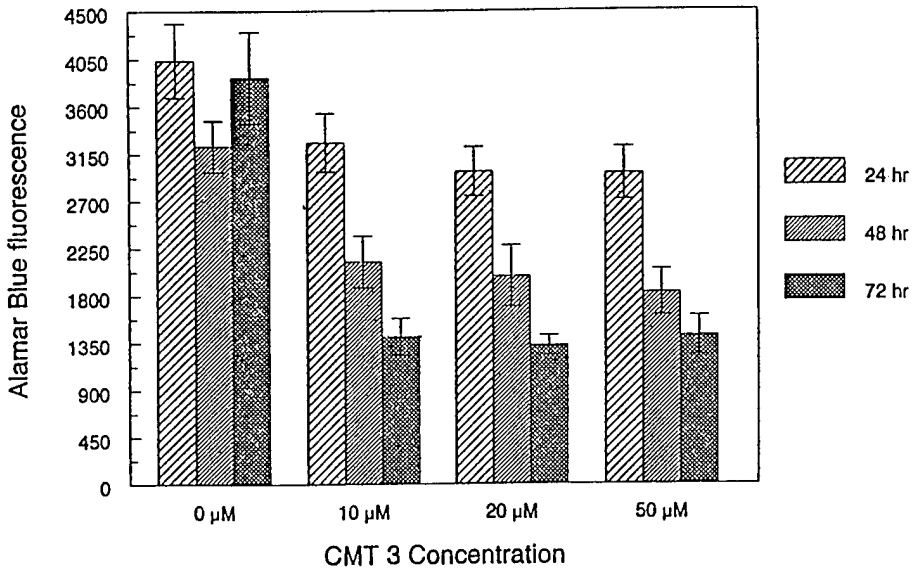


Figure 12

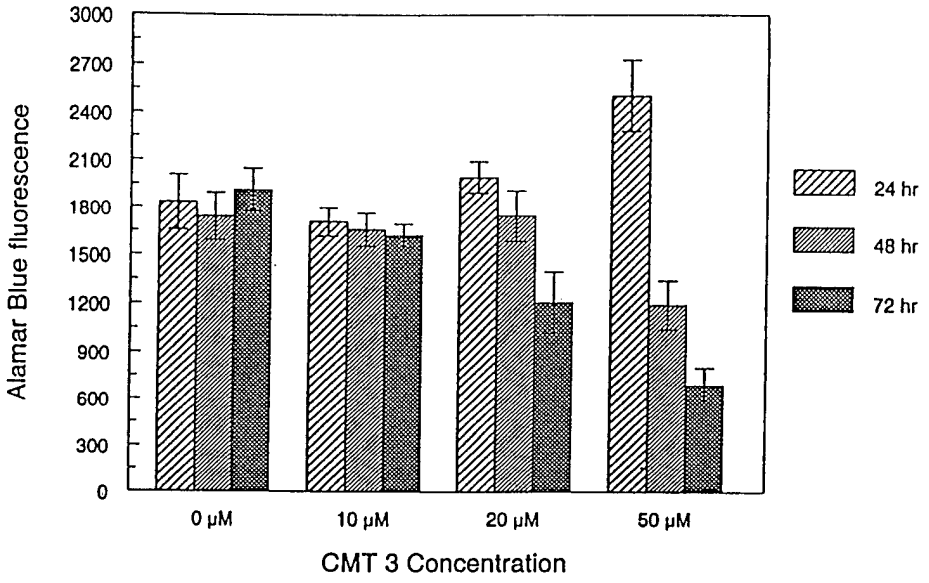
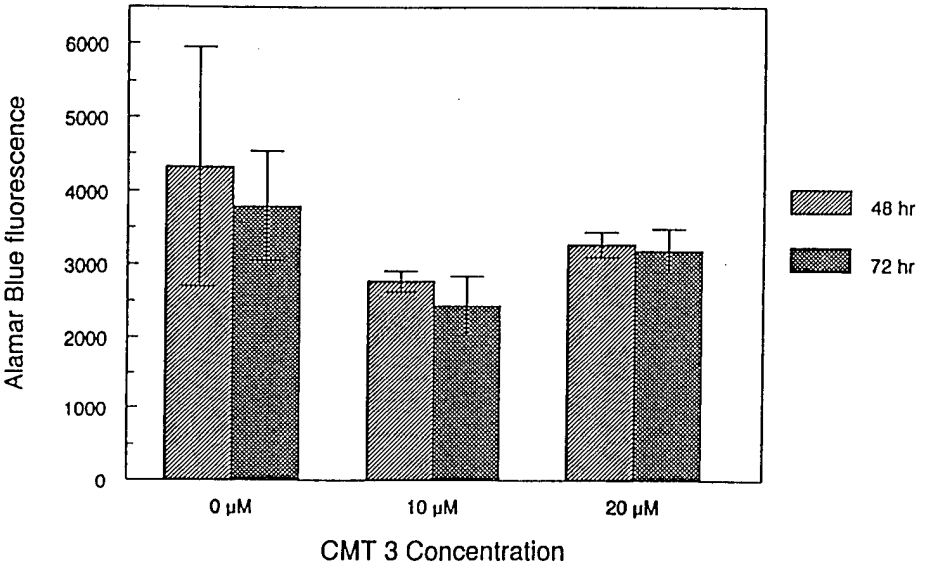
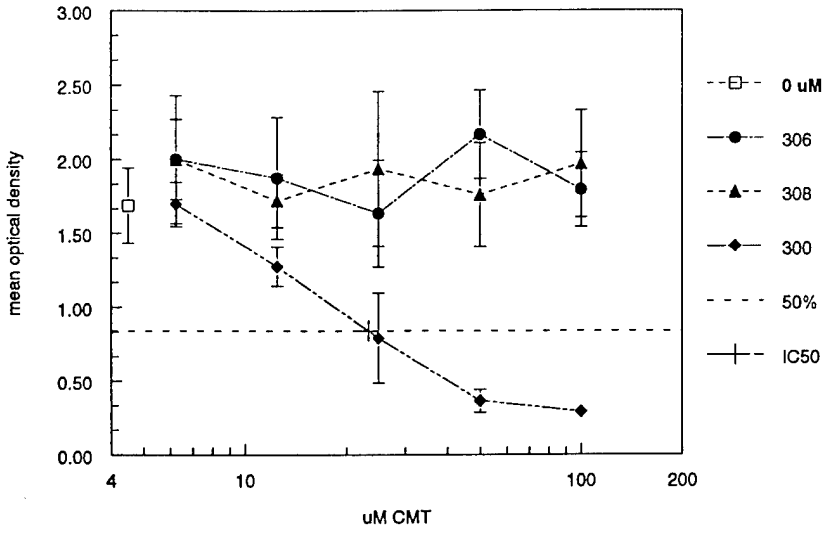


Figure 13



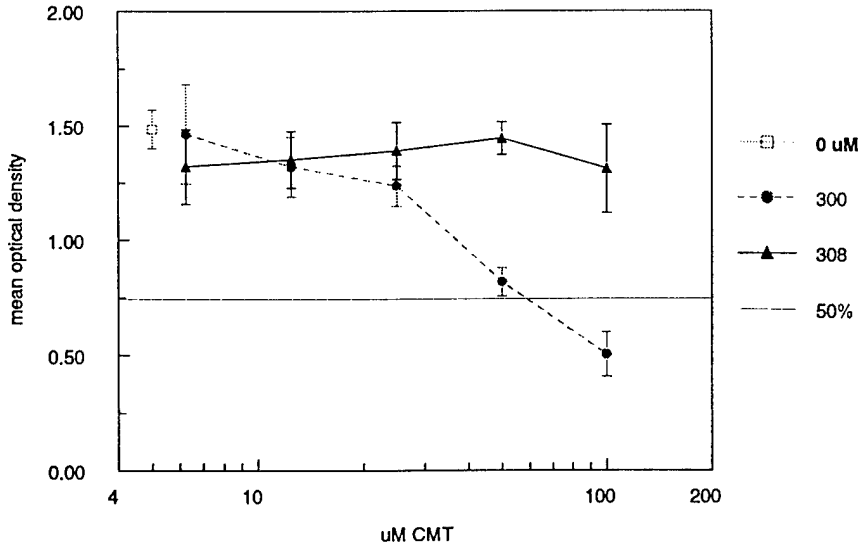
Effect of CMTs on Normal Prostate Epithelium

Figure 14



Effect of CMT 300 & 308 on Normal Prostate Stromal Cells

Figure 15



Joint Symposia

JS-1

Cysteine protease inhibitors as antimalarial drugs. Philip J. Rosenthal, Bhaskar Shenai, Chi Chang, Wendy Kang, and Andrey Semenov. Dept. of Medicine, San Francisco General Hospital and University of California, San Francisco, CA, USA 94943-0811. e-mail: rosnthl@itsa.ucsf.edu.

The *Plasmodium falciparum* cysteine protease falcipain is required for the degradation of hemoglobin by erythrocytic malaria parasites. This protease appears to act cooperatively with the aspartic proteases plasmepsins I and II and perhaps other proteases to hydrolyze hemoglobin. Characterization of affinity-purified falcipain has shown that a reducing environment is required for maximal hemoglobinase activity and that clear differences exist in substrate and inhibitor specificity between parasite and host cysteine proteases. Peptidyl fluoromethyl ketone and vinyl sulfone inhibitors of falcipain blocked hemoglobin degradation and development by cultured parasites, and selected compounds cured *Plasmodium vinckei*-infected mice. One potent inhibitor, N-methyl piperazine urea-leucine-homophenylalanine-2-naphthalene vinyl sulfone, cured about 40% of mice when administered orally twice-a-day for four days. We have also evaluated the antimalarial effects of combinations of cysteine and aspartic protease inhibitors. When incubated with cultured *P. falciparum* parasites, cysteine and aspartic protease inhibitors exhibited synergistic effects in blocking parasite metabolism and development, and this effect appeared to be due to the synergistic inhibition of plasmodial hemoglobin degradation. When evaluated for the treatment of murine malaria, a combination of a vinyl sulfone falcipain inhibitor and the aspartic protease inhibitor pepstatin was much more effective than higher concentrations of either compound used alone. Our results support a model whereby plasmodial cysteine and aspartic proteases participate in the degradation of hemoglobin and suggest that inhibitors of falcipain and other plasmodial proteases have promise as antimalarial chemotherapeutic agents.

JS-2

How Does Blood Feeding Regulate the Synthesis of Trypsin in the Mosquito Midgut? MICHAEL A. WELLS, Department of Biochemistry and Center for Insect Science, University of Arizona, Tucson, AZ, 85721, mawells@u.arizona.edu

Ingestion of a blood meal induces two phases of trypsin synthesis in the midgut of *Aedes aegypti* females. The first phase, which encompasses the first 4-6 hours following a blood meal, is characterized by the presence of small amounts of early trypsin. The second phase, which occurs between 8 and 36 hours after blood feeding, is characterized by the presence of large amounts of late trypsin. A specific form of regulation of trypsin synthesis characterizes each phase: early trypsin synthesis is regulated at the translational level, while late trypsin synthesis is regulated at the transcriptional level. Transcription of the early trypsin gene starts a few hours after adult emergence and is under control of juvenile hormone. However, the early trypsin mRNA is stored in the midgut epithelium and remains untranslated until a blood meal is taken. The exact mechanism responsible for initiating translation is presently unknown, but an increase in the size of the amino acid pool in the midgut is sufficient to activate translation of early trypsin mRNA. The enzymatic activity of early trypsin plays a unique and critical role in the regulation of late trypsin synthesis. Early trypsin acts like a sensor to determine whether protein has entered the midgut. It carries out limited proteolysis of the ingested proteins and, somehow, the products of this limited proteolysis induce transcription of the late trypsin gene. Late trypsin is the protease responsible for the majority of the endoproteolytic cleavage of the meal proteins. Once transcription has been activated, the extent of transcription of the late trypsin gene is proportional to the amount of protein present in the meal. In addition, the amount of late trypsin protein translation is controlled by the amount of amino acid released during digestion of the meal proteins. Regulation at both transcriptional and translational levels allows the midgut to adjust the amount of late trypsin with remarkable flexibility in response to a particular meal. Supported by NIH Grant AI31951.

JS-3

Proteases and integrins associated with invadopodia of malignant and tissue cells: Expression of the invasive phenotype and collagen remodeling at sites of the active cell surface. Wen-Tien Chen, Ph.D., Department of Medicine/Medical Oncology, SUNY at Stony Brook, New York 11794-8160. Email: wchen@mail.som.sunysb.edu

Proteases and integrins may provide malignant cells the ability to break out of tissue barriers and normal cells to heal. Various membrane-bound proteases, including serine- and metallo-types, and the cell adhesion molecule integrins participate in expression of the invasive phenotype and collagen remodeling at sites of the active cell surface. Such surface structures are in the form of invadopodia or lamellipodia, which contact and remodel the extracellular matrix at the leading edge of the migratory cell. Recent evidence suggests two distinct, proteolytic localization systems at invadopodia. The localization and activation of Matrix Metalloproteinases (MMP) appear to involve the association of tri-molecular complex, 72-kDa MMP-2 (Gelatinase A), tissue inhibitor of MMP-2 (TIMP-2) and MT1-MMP, with the actin cytoskeleton. Other cell surface peptidases involved in invadopodial activities are the serine integral membrane proteases (SIMP) seprase and dipeptidyl peptidase IV (DPPIV). In human malignant melanoma cell line LOX, a specific integrin, alpha3 beta1, docks seprase and localizes its proteolytic activity at invadopodia that may allow the cell to break alpha5 beta1 integrin-matrix adhesion. In wound-activated endothelial cells and fibroblasts, as well as in human breast carcinoma cell lines MDA-MB-436 and Hs578T, alpha3 beta1 integrin drives seprase/DPPIV heteromeric complex to sites of matrix degradation and collagen turnover. We suggest that invadopodium-associated proteases are ideal targets for the diagnosis and treatment of cancer as their presence in association with activated malignant and tissue cells may signal increased metastatic potential.

JS-4

Novel Inhibitors in Inflammation and Metastasis. S.R.SIMON, E.J. Roemer, W. Bellucci, Y. Gu, Q.L. Ying, C. Mannino, and E. Spero. Pathology Dept, SUNY at Stony Brook, Stony Brook, NY 11794-8691. E-mail: SSIMON@PATH.SOM.SUNYSB.EDU.

Agents to inhibit destruction of connective tissue by inflammatory cells and invasive tumor cells should ideally target activities of multiple classes of proteinases. Endogenous antiproteinases with specificities against individual proteinases may show impressive potency when studied in isolation, but the synergistic actions of multiple proteinases, along with other components of the inflammatory response or the invasive phenotype, may inactivate these endogenous defenses. Inhibitors of multiple proteinases should be especially effective under these circumstances, blocking pathologically excessive proteolysis and protecting endogenous antiproteinaes. We have been evaluating several chemically modified tetracyclines (CMTs) which possess pleiotropic inhibitory activities, using a series of in vitro assays in which the action of multiple proteinases can be monitored. A major tool in these assays has been the use of radiolabeled complete interstitial extracellular matrices as substrates for degradation by inflammatory cells, tumor cells, or their conditioned media. The most promsing of the CMTs inhibit matrix metalloproteinase (MMP) activity as well as activity of the serine proteinase neutrophil elastase (NE). Screening of inhibitory potencies employs a novel coupled amidolytic assay for MMP inhibition with prourokinase as an MMP substrate, and a dye-binding assay for elastinolysis in addition to the classical amidolytic assay for inhibition of NE activity. These assays are all adaptable to use with inflammatory or tumor cells as well as purified proteinases and can be modified to introduce components which are present in the microenvironment of acute inflammatory foci or invasive tumors and which may alter the effectiveness of synthetic antiproteinases. The distinct specificities of synthetic and endogenous antiproteinases are used in the assays to evaluate the capacity of synthetic inhibitors to protect endogenous antiproteolytic defenses. (Supported by NIDR [DE-10985], STRC, USAMRMC, Collagenex Pharm., and SUSB Biotechnology Center)

VT-1021

A New Screening Assay for the Evaluation of Inhibitors of Matrix Metalloproteinases. W.J. BELLUCCI, S.R. Simon and E.J. Roemer, Department of Pathology, SUNY Stony Brook, Stony Brook, NY, 11794-8691. E-Mail: wbellucc@yahoo.com

Matrix metalloproteinases(MMP's) are a family of zinc dependent endopeptidases which play important roles in tissue remodeling and wound healing. Pathologies known to be associated with increased MMP activity include arthritis, atherosclerotic lesions and more recently, tumor cell invasion and metastasis. The endogenous MMP inhibitors or tissue inhibitor of metalloproteinases (TIMPS), have short half-lives which limit their pharmacological applications. Therefore, the development of exogenous MMP inhibitors has become a major interest of pharmaceutical research, resulting in a growing need to develop screening procedures for potential inhibitors of MMP's. By modifying a BIOTRAK MMP-9 ELISA assay we are able to measure the activity of MMP-9 as reflected by the generation of the catalytically active two chain form of urokinase which is formed by MMP-9 mediated proteolysis. Continuous recording of absorbance over a four hour period yields a distinctive exponential curve, the kinetic constant for which is a measure of MMP-9 activity. Our modified protocol involves initial pre-incubation of the active MMP-9 with potential MMP-9 inhibitors prior to further incubation with single chain urokinase and a chromogenic urokinase substrate. The rate of active urokinase formation was determined in the presence of EDTA, a known MMP inhibitor, as well as different doses of chemically modified tetracyclines(CMTs) being evaluated for pharmaceutical applications. A dose of 5mM EDTA produced complete inhibition, while the CMTs showed a range of inhibitory potencies. Neither EDTA nor the CMTs had any direct effect on hydrolysis of the urokinase substrate by two chain urokinase which had been previously generated from its single chain form by MMP-9 in the absence of MMP-9 inhibitors. This new assay offers promise for reproducible and efficient screening of MMP-9 inhibitors. (Supported by NIDR[R01-DE10985], STRC, CFF, Collagenex Pharmaceuticals, Inc., and SUSB Biotechnology Center)

VT-1022

Enhanced Myometrial Secretion of Parathyroid Hormone-Related Protein (PTHrP) during Pregnancy is not Mediated by Estrogen. P. DIXON, W. Barth, and J.S. Schenberger. Clinical Investigations, Wilford Hall Medical Center, Lackland AFB, TX 78236-5300. E-mail: jedielni@hotmail.com

In animals, the smooth muscle relaxant PTHrP increases during pregnancy presumably through an effect of estrogen. Although PTHrP is present in the human myometrium, its role in parturition has not been studied. Thus, we examined the ability of human myometrial cells to secrete PTHrP and attempted to reproduce a pregnant secretory pattern with estrogen. Biopsies obtained from term, pregnant women (P;n = 11) and non-pregnant women(NP;n = 8) were digested with collagenase and cultured in 10% serum. Cultures were treated with TGF-β1(0-10 ng/ml) to stimulate PTHrP secretion. 3 NP samples treated with 17β-estradiol were analyzed for basal and TGF-β1-stimulated PTHrP secretion. PTHrP levels were determined by RIA. Trends were analyzed using ANOVA. We found that TGF-β1 increased PTHrP secretion in a dose-dependent manner ($p < 0.001$). Pregnancy also increased PTHrP secretion over time ($p<0.05$) with maximal increases noted at 24 hrs (P:1780±270 vs. NP: 1150±200 fmol/mg protein, $p<0.01$). The addition of 17β-estradiol to NP samples did not alter the basal or TGF-β1-stimulated PTHrP secretion. We conclude that pregnancy increases the capacity of human myometrial cells to secrete PTHrP. This effect cannot be duplicated simply by treating NP tissue with estrogen. Thus, while our findings support the concept that PTHrP promotes uterine relaxation in the presence of a growing fetus, the process does not appear to be secondary to estrogen.

VT-1023

Electrical Stimulation Decreases the Coupling Efficiency Between beta-Adrenergic Receptors and Cyclic AMP Production in Cultured Muscle Cells. R.B. YOUNG and K.Y. Bridge. Marshall Space Flight Center, ES76, Huntsville, AL 35812. E-mail: ronald.young@msfc.nasa.gov.

Electrical stimulation of skeletal muscle cells in culture is an effective way to simulate the effects of muscle contraction and its effects on gene expression in muscle cells. Expression of the beta-adrenergic receptor and its coupling to cyclic AMP synthesis are important components of the signaling system that controls muscle atrophy and hypertrophy, and the goal of this project was to determine if electrical stimulation in a pattern simulating slow muscle contraction would alter the beta-adrenergic response in muscle cells. Chicken skeletal muscle cells that had been grown for seven days in culture were subjected to electrical stimulation for an additional two days at a pulse frequency of 0.5 pulses/sec and a pulse duration of 200 msec. At the end of this two-day stimulation period, beta-adrenergic receptor population was measured by the binding of tritium-labeled CGP-12177 to muscle cells, and coupling to cyclic AMP synthesis was measured by RIA after treating the cells for 10 min with the potent bAR agonist, isoproterenol. The number of beta adrenergic receptors and the basal levels of intracellular cyclic AMP were not affected by electrical stimulation. However, the ability of these cells to synthesize cyclic AMP was reduced by approximately 50%. Thus, an enhanced level of contraction reduces the coupling efficiency of beta-adrenergic receptors for cyclic AMP production. (Supported in part by Lilly Research Laboratories).

VT-1024

Effect of Matrix, Medium Composition, and Constant Medium Flow on Viability and Functionality of Hepatocytes in Culture. J.L. SMITH and E.L. LeCluyse. School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599. E-mail: jessmith@email.unc.edu.

The OBJECTIVE of this study was to determine the effect of matrix, medium composition and constant medium flow on the viability and functionality of hepatocytes in culture. METHOD: Hepatocytes were cultured on a rigid collagen substratum (UR) or in a gelled collagen sandwich (SG) and were maintained with Dulbecco's Modified Eagle Medium (DMEM) or Modified Chee's Medium (MCM) under static conditions or on a rocker table. Cells were monitored over two weeks in culture for lactate dehydrogenase (LDH) leakage, albumin secretion and intracellular glutathione (GSH) content. In addition, hepatocytes were treated with prototypical inducers of Cytochrome P450 (CYP) 1A and 2B, beta-naphthoflavone and phenobarbital, respectively. RESULTS: LDH leakage revealed enhanced recovery after initial plating in cells maintained with MCM vs. DMEM regardless of kinetic condition. Further, UR cultures exhibited lower cell viability at two weeks than SG cultures. Cultures exhibited a matrix effect (SG greater than UR), but no kinetic effect with respect to albumin secretion. GSH content remained similar across groups over time in culture; however, cells in the UR configuration exhibited a surge of GSH on day 4 in culture, regardless of kinetics. CYP1A induction response was similar among static culture conditions; however, under kinetic conditions, cells in the UR configuration exhibited a greater induction response than cells maintained as SG. CYP2B induction response was greater in SG vs. UR, in MCM vs. DMEM, and under constant motion vs. static conditions. CONCLUSION: Constant medium flow appears to enhance the differentiation and viability of hepatocytes maintained in the SG configuration while perpetuating the dedifferentiation of hepatocytes in the UR configuration.

VT-1029

Evaluation of the Cytotoxicity of Chemically Modified Tetracyclines (CMT) on LNCaP Prostate Tumor Cells. S.Boumakis, S.R. Simon and E.J. Roemer, Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY, 11794-8691

The major source of morbidity and mortality of prostate cancer is due to the invasive and metastatic potential of the tumor cells. Invasiveness may be linked to expression of one or more members of the family of matrix metalloproteinases (MMPs). Other proteinases, such as the serine proteinases, may also participate in tumor cell invasion and degradation of tissue stroma. We use LNCaP prostate tumor cells in *in vitro* assays to evaluate the capacity of chemically modified tetracyclines with multiple antiproteolytic activities to inhibit tumor-mediated tissue destruction. We have employed an *in vitro* system based upon a complete interstitial extracellular matrix (ECM) synthesized by rat heart smooth muscle cells (R22s), on which LNCaP cells can be cultured, to model tumor-mediated tissue degradation. Before undertaking studies on tumor cell-mediated matrix degradation, optimal LNCaP culture conditions were characterized. Preliminary studies determined which medium formulations, plating densities and feeding schedules best met LNCaP growth requirements, as well as the time required for the cells to reach confluence. LNCaP cells appear to be unusually sensitive to cytotoxic action of the CMTs, and may be useful targets for establishing whether this family of drugs may be selectively tumoricidal. Cytotoxicity assays which address this additional mode of CMT action against tumor cells help identify candidate compounds for further study. [funded by USAMRMC, Collagenex Corp, and NSF RAIRE Grant No. STI 9620074]

VT-1030

Effects of Chemically Modified Tetracyclines (CMTs) on Colon and Breast Cancer Cells *In Vitro*. L.C. MUSACCHIA, Y. Gu, E.J. Roemer, S.R. Simon. Departments of Pathology and Biochemistry, State University of New York at Stony Brook, Stony Brook, NY 11794-8691. lmusacch@ic.sunysb.edu.

The complexity in defeating cancer exists in its metastatic capability. With this in mind our approach to tackling this disease has for some time been directed towards searching for a compound that will be effective in treating cancer clinically. Experiments were performed on breast and colon cancer cell lines to determine the cytotoxic and enzymatic inhibitory effects of chemically modified tetracyclines (CMTs), which have been proven to inhibit metalloprotease (MMP) activity in periodontal diseases. Using MTS, a tetrazolium salt, as our cytotoxic detector, we found that CMT-3, CMT-5, CMT-8, and the tetracycline derivative DOXY were non-toxic to the breast cancer cell to a concentration of up to 20μM. Conversely, all these compounds, except for CMT-5, were cytotoxic to the colon cancer cell at concentrations as low as 5μM, with CMT-3 being the most cytotoxic. Using our *in vitro* model of radiolabeled extracellular matrix (ECM) on multi-well plates, we found that CMT-3 does affect these cells by inhibiting their degradation of the ECM at concentrations of 50μM and 100μM by about 25 and 40 percent, respectively. 1,10-phenanthroline (OP), a known MMP inhibitor, was also found to protect the ECM from breast cancer cell activity. Breast cancer cells are known to produce mainly gelatinase MMP-9. A gelatinase assay showed that MMP-9 activity was inhibited by 15 and 50 percent at CMT-3 concentrations of 10μM and 20μM, respectively. This indicates that CMT-3 is involved in protecting the ECM from MMP-9 activity. (Supported by NIDR [R01-DE10985], STRC, CFF, Collagenex Pharmaceuticals, Inc., and SUSB Biotechnology Center.)

VT-1031

Chemically modified tetracycline 300 and its derivatives inhibit extracellular matrix degradation by polymorphonuclear leukocytes and Cystic Fibrosis sputum. B. Sehgal, S.R. Simon, E.J. Roemer. Department of Pathology, SUNY Stony Brook, Stony Brook, NY, 11794-8691

Tissue destruction in inflammatory diseases ranging from arthritis to cystic fibrosis is mediated by several proteinases released by neutrophils, including matrix metalloproteinases (MMP) which help initiate collagen destruction, and serine proteinases, such as elastase, which attack most other components of extracellular matrix. A number of chemically modified, non-antimicrobial tetracyclines (CMTs), inhibit matrix metalloproteinases, and in some cases block the activity of neutrophil elastase as well. CMT 300 through 311 were tested for this broad spectrum inhibitory activity using an in vitro assay based upon degradation of a native interstitial extracellular matrix (ECM) produced by R22 rat heart smooth muscle cells. This ECM, consisting of type I collagen, elastin, proteoglycans, and fibronectin, can be radiolabeled by growing the cells in medium supplemented with [^{3}H]-proline or fucose, and [^{35}S]-sulfate. Neutrophils (PMN) isolated from human donor blood were placed on the ECM, stimulated with lipopolysaccharide(LPS), and incubated with 50, 100, 200, and 300 μM concentrations of CMTs. The same range of CMT concentrations was also incubated on ECM with sputum isolated from cystic fibrosis patients in place of LPS-stimulated PMN. Degradation of collagen, glycoproteins, and proteoglycans was quantified by analyzing the fraction of total incorporated radiolabels released from ECM after exposure to PMN or sputum. It was found that some CMTs can reduce degradation of ECM by almost 40-50% in a dose dependent fashion without significant cytotoxicity. CMTs may eventually be developed into new drugs designed to treat excessive tissue destruction associated with many inflammatory diseases. (Supported by NIDR [R01-DE10985], STRC, CFF, Collagenex Pharmaceuticals, Inc., USB Biotechnology Center)

VT-1032

The effect of chemically modified tetracyclines on the inhibition of sputum elastase in the presence of polyanions. E.F. SPERO, E.J. Roemer, S.R. Simon and C. Ren, Department of Pathology, SUNY Stony Brook, NY, 11794-8691, ESPERO@IC.SUNYSB.EDU

Current studies suggest that tetracyclines which do not exhibit antimicrobial properties may have other therapeutic effects. Such chemically modified tetracyclines (CMTs) were studied as inhibitors of the protease elastase present in the lungs of cystic fibrosis patients. The lung environment was modeled *in vitro* by the addition of polyanions such as DNA, mucin and alginate which is similar to the polysaccharide coat of *Pseudomonas aeruginosa* , a common bacterial pathogen. Inhibition constants for the CMTs were established through Dixon plot analysis of assays conducted at various substrate concentrations in the presence of each of the individual polyanions. The effect of fragmented DNA on elastase activity was also studied by the addition of DNAase, a recent treatment for cystic fibrosis. The elastase activity (amidolytic rate) was increased significantly in the presence of fragmented DNA, even with the addition of inhibitors. Concentrations of polyanion were then lowered to physiologically relevant levels, as well as combined. The effectiveness of several of the inhibitors was enhanced by the addition of polyanions. The prospect of an elastase inhibitor which could remain effective in the cystic fibrosis lung environment may be a useful treatment for the disease. (Supported by NIDR [RO1-DE10985], Collagenex Pharmaceuticals, Inc.)

VT-2004

Selective expansion and non-radioisotopic assay of human natural killer cells. S. Watanabe, H. Harada, K. Saijo, T. Ohno. RIKEN (Inst. Physical. & Chem. Res.) Cell Bank, E-mail: satoru@rtc.riken.go.jp

We developed a simple method for human natural killer (NK) cell expansion with an adhesive target cell line HFWT that was derived from human Wilm's tumor. HFWT cells are scarcely expressing MHC-class I and class II molecules and therefore highly sensitive to human NK cells. Culture of peripheral blood mononuclear cells (PBMC) of healthy volunteers on HFWT cells for 10–16 days resulted in selective expansion of NK (CD3-CD56+CD16+) cells. PBMC, initially contained less than 15% of NK cells, were shared more than 50% of the population by NK cells after the culture. Cr-51 release assay has long been used as the standard method to quantify activity of NK cells. To avoid use of the radioactive substance in the assay, the adhesive HFWT cells were stained with crystal violet (CV) before and after the 4-hr incubation with NK population in which CD3-CD56+CD16+ cells shared 57%. NK cells were washed off after the incubation. The dye were quantified by the absorption at 570nm. At the effector/target ratio of 2 and 8, surviving target HFWT cells were 67% and 9%, respectively. More precise dose response curve corresponded reversively to that obtained by the standard Cr-51 release assay. After the 24-hr incubation, higher sensitivity of the killing was detected in the CV assay. These results suggest that human NK cells and non-radioisotopic CV assay are useful for further application in tumor therapy and/or health monitoring.

VT-2006

Two-dimensional Cell Blot Method. T. TERASAKI, Z. Yamaizumi and K. Tanaka. Pathol., Biol. and Radiobiol. Div., National Cancer Center Research Institute, Tsukiji 5-1, Chuo-ku, Tokyo 104-0045, Japan Email: tterasak@ncc.go.jp

In order to characterize functional proteins that induce changes in cells, a new method (Two-dimensional Cell Blot Method) was established. Fibronectin was partially digested with trypsin, two-dimensionally electrophoresed, transferred to a membrane and stained. After photocopied, the membrane was de-stained, blocked and baby hamster kidney cells (BHK-21) were cultured on the membrane over night. Cells on the membrane were fixed, stained and observed macroscopically or microscopically. Many protein spots to where BHK-21 cells attached and many protein spots to where those cells did not attach were observed. Immunological studies using antibody that had the activity to react with fibronectin cell binding domain showed that all the spot to where BHK-21 cells attached reacted with this antibody and all the spot to where those cells did not attach did not react with this antibody. Amino acid sequences of the proteins that are separated by two-dimensional polyacrylamide gel electrophresis and transferred to the membrane can be determined using amino acid sequencer. These showed that two-dimensional cell blot method is useful to characterize functional proteins that have activities to bind cells. This method must be also useful to characterize functional ptroteins that have activities such as to induce cell morphological changes, cell changes that can be detected by specific antibodies or cell death.

VT-2007

Role Of Matrix Metalloproteinases In Alpha-1-Proteinase Inhibitor Degradation By Neutrophil-Derived Proteases. W.J. BELLUCCI, E.J. Roemer, C.L. Ren and S.R. Simon, Dept. of Pathology, SUNY Stony Brook, Stony Brook, NY, 11794-8691. E-Mail: wbellucc@yahoo.com

Alpha-1 Proteinase Inhibitor (a1PI), the endogenous inhibitor of serine proteases is found proteolytically inactivated in diseases resulting from a recurrent inflammatory response such as Emphysema and Cystic Fibrosis. The goal of this study was to investigate the potential role matrix metalloproteinases (MMPs) might play in the degradation of a1PI. We lysed freshly isolated polymorphonuclear neutrophils (PMNs) in the presence or absence of 2.5mM Phenylmethylsulfonyl Fluoride (PMSF), a serine protease inhibitor. a1PI was incubated for 24 hours with normal or PMSF treated lysate with or without MMP inhibitors such as EDTA and 1,10 phenanthroline. SDS-PAGE showed degradation of a1PI by the normal lysate, which could be inhibited by MMP inhibitors. Degradation was also inhibited by incubation with PMSF or pre-treatment of PMN lysates with PMSF. Gelatin zymography showed active MMP 2 and 9 in the normal lysate whereas the inactive proforms were observed in PMSF pretreated lysates. Inhibition of in vitro extracellular matrix degradation by a1PI was enhanced by PMSF, EDTA and 1,10 phenanthroline. We conclude that MMP activity is involved in the degradation of a1PI and hypothesize that a serine protease activity may be required for this MMP activity. This study was supported by NIH (NIDER) DE-10985 (431-0264A)%llaGenex Pharmaceuticals, Inc., (431-6087A); USAMRMCDA-MD-1798-18560 (431-1241A); SUSB Center for Biotechnology (NYS)Science & Technology Foundation) (431-X324Q)

VT-2008

Evaluation of the Composition of the Extracellular Matrix Synthesized by Human Prostate Stromal Cells in Culture. E. SCOTTO-LAVINO, H.A. Sawka, S.R. Simon and E.J. Roemer, Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY 11794-8691 E-mail: escottol@ic.sunysb.edu

Extracellular matrix (ECM) synthesized by R22 rat heart smooth muscle cells *in vitro* has been used in our laboratory to study the behavior of certain cell lines. We have adapted the culture protocols currently used for the rat cell system to establish the appropriate parameters for a human model based on commercially available normal human prostate stromal cells. These prostate cells grow very well in both a proprietary serum-free culture medium and in medium containing 5% FBS. With the addition of ascorbic acid, they will synthesize a stable, fibrous ECM. Culture medium containing either [^{3}H] proline and [^{35}S] sulfate or [^{3}H] fucose and [^{35}S]-cysteine/methionine is used to selectively radiolabel ECM components. Once the ECM has grown, the cells are removed and the matrix composition is analyzed by sequential enzymatic degradation. Individual enzymes: heparinase I & III, trypsin, chondroitinase, collagenase and human leukocyte elastase; are each incubated with the ECM for twenty-four hours, to extract individual matrix components. Supernatants from each step of the sequence are read by scintillation counting and analyzed to determine the relative proportions of components in the original, intact ECM. Enzyme-free control wells for each sample are incubated with the appropriate buffers and analyzed for spontaneous degradation of matrix, providing a relative measure of the ECM's physical stability and strength. By comparing matrix synthesized by cells from several different donors, we hope develop an *in vitro* human matrix system to use as a substrate for study of the degradative behavior of human prostate tumor cells. This study was supported by NIH(NIDER) DE-10985 (431-0264A); Colla-Genex Pharmaceuticals, Inc., (431-6087A); USAMRMC DA-MD-1798-18560 (431-1241A); SUSB Center for Biotechnology (NYS Science & Technology Foundation) (431-X324Q) and URECA Grant #264900.

VT-2017

Expression of MHC Antigens and Adhesion/Costimulation Molecules of Dendritic Cells from Human Blood During their Differentiation *In Vitro*. M. CHIRIVA-INTERNATI[1], F. Grizzi[2], G. Ceva-Grimaldi[3] and N.Dioguardi[2]. [1] Myeloma and Transplantation Research Center, Arkansas Cancer Research Center, UAMS, Little Rock, Arkansas, USA; [2]Scientific Direction, Istituto Clinico Humanitas, Rozzano, Milano, Italy; [3]Department of Pathology, Istituto Clinico Humanitas, Rozzano, Milan, Italy.E-mail: fabio.grizzi@humanitas.it

Dendritic cells (DC) are highly specialized antigens presenting cells present in peripheral lymphoid and non-lymphoid organs. DC are also present in the thymus, where they serve to eliminate potentially autoreactive cells from the T-cells repertoire. The major characteristic qualifying DC as professional antigen presenting cells is their high expression of MHC antigens and adhesion/co-stimulation molecules. Since both macrophages (M) and DC can differentiate from blood monocytes, depending on the different culture conditions, we investigated their kinetics of expression of MHC antigens and several adhesion/co-stimulation molecules. 42 ml of peripheral blood from three healthy volunteers was centrifuged on Ficoll-Hypaque to obtain PMBC. These cells were then plated (1x107/3 ml per well) in AIM-V culture medium. After 2 h at 37° C, non-adherent cells were removed, and the adherent cells were cultured at 37° C in a humidified 5% CO2/95 % air incubator, in medium supplemented with recombinant human GM-CSF (800 U/ml), and IL-4 (1000 U/ml). Cells were evaluated for surface marker expression using FACS analysis at different time points [i. e. time 0, day 5, day 7 and three days further in the presence of TNF-a (day 10)]. For each time point, a panel of mAbs recognizing the following antigens was used: CD 40, CD 54, CD 80, CD1a, CD 86, CD 14 and CD83. MHC Class I and MHC Class II were highly expressed in DC. CD 80, CD 86 and CD 54 expressions increased significantly until day 10, while CD 1a remained stable throughout the culture period. M cells maintained up-regulated CD 86, whereas CD1a declined throughout the culture period. CD 54 remained highly expressed in M, becoming undetectable by day 5 whereas CD 40 was transiently expressed on M until day 5. Our results show the feasibility in analyzing the different patterns in surface antigens on human DC. These qualitative and quantitative immunologically differences in respect to other cells, such as M, enable their different properties in naive T cell priming.

VT-2018

CYTOTOXIC EFFECTS OF CHEMICALLY MODIFIED TETRACYCLINES ON R22 CELLS. KIMBERLY GUILFOY, Sanford R. Simon and Elizabeth J. Roemer, Department of Pathology, SUNY Stony Brook, Stony Brook, NY 11794-8691 E-mail: kguilfoy@ic.sunysb.edu

Many cancer treatments affect not only tumor cells, but normal body cells as well. In vitro assays for potential cancer treatments are therefore used to help determine a potential medication¡s effect on both of these types of cells. Ideal treatments should show the greatest cytotoxic effects on tumor cells, while normal cells experience minimal damage. Using in vitro cytotoxicity assays on rat heart smooth muscle cells (R22) enables chemically modified tetracyclines (CMTs) to be screened for their cytotoxic effects on a ¡°normal¡± cell. The R22 cells are treated with the CMTs at both confluent and sub-confluent densities. This permits screening of both mitotic and quiescent cells, and thus roughly models representing the varying levels of mitotic activity seen in different tissue types. A cytotoxic dose response to the CMTs is the primary purpose to this particular assay. MTS, a tetrazolium salt, was used as an indicator of the CMTs cytotoxic effect. The MTS indicator, which is normally yellow, becomes brown when reduced by the viable R22 cells. Therefore, the darker the color, the greater the number of surviving cells. The optical density produced by the color change is measured, and the IC50 calculated for each of the CMTs. The IC50 provides the concentration of each CMT at which half of the cells are dead. Visual observations determined the morphological effects the CMTs had on the R22 cells. This assay; along with numerous additional cytotoxic assays; is being used to help determine which CMTs have the best potential as cancer treatments. This study was supported by NIH(NIDER) DE-10985 (431-0264A); CollaGenex Pharmaceuticals, Inc., (431-6087A); USAMRMC DA-MD-1798-18560 (431-1241A); SUSB Center for Biotechnology (NYS Science & Technology Foundation) (431-X324Q) and URECA Grant #264900.

VT-2019

Biochemical Changes Induced in Human Cells by the Vesicating Agent Sulfur Mustard. W.J. SMITH, O.E. Clark, F.M. Cowan, M.E. DeJoseph and C.L. Gross. Pharmacology Division, US Army Medical Research Institute of Chemical Defense, APG, MD 21010-5400. E-mail: WILLIAM.SMITH@AMEDD.ARMY.MIL

Development of medical countermeasures against blistering agents requires definition of the pathophysiology produced by these agents. Sulfur mustard (HD) is a genotoxic agent that causes severe skin blistering with associated inflammatory responses. We studied three pathways of cell injury: enzyme activation, intracellular ion changes, and inflammatory mediator induction. We demonstrated time- and dose-dependent activation of poly(ADP-ribose) polymerase (PARP) in HEK. At 10 mM mustard there is a significant elevation of PARP activity by 4 hours postexposure. At 100 mM, elevated PARP was detected at 2 hours postexposure. PARP activity decreased to baseline by 6 hours and then returned with a 30% elevation at 24 hours. Our routine culture conditions using passage 3 HEK showed no large changes in intracellular calcium after HD exposure, but use of early (passage 2) HEK grown on coverslips demonstrated a low level (<20%) increase in intracellular calcium within 15 minutes of HD exposure. Expression of Fc receptors by HEK was increased following HD, as was binding of the initial complement sequence component, C1q. The interleukins IL-6 and IL-1b were only seen at the highest concentration of HD, 300 mM (a highly toxic dose). IL-8 was increased in supernates of HEK exposed to 50, 100 and 300 mM. While each pathway demonstrated some HD-induced changes, neither the extent nor the timing of the changes suggests a single mechanism of cell injury.

VT-2020

Phototoxicity Testing Using 3-D Reconstructed Human Skin Models. P.A. JONES, A.V. King and L.K. Earl, SEAC Toxicology Unit, Unilever Research Colworth, Colworth House, Sharnbrook, Bedford, UK, MK44 1LQ. Email: penny.jones@unilever.com

The potential phototoxic hazard of materials is currently assessed in-house using a tiered testing strategy involving *in vitro* assays, including the validated 3T3 cell neutral red uptake phototoxicity test. However, a second test is required to allow confirmatory testing of materials of low aqueous solubility, prior to their use in products. The purpose of this study was to investigate if an *in vitro* 3-D reconstructed human skin model, EpiDerm™ (MatTek Corporation, USA) could be used to identify the phototoxic hazard of such test materials. The assay was based on the methods of Liebsch *et al* (ALTEX, **14**, 165, 1997) but with some differences. A UVB filter was used instead of a UVA filter and organic solvents were used as the dosing vehicles instead of water or sesame oil. 8 chemicals of known *in vivo* phototoxicity and 4 ingredients were assayed. EpiDerm™ cultures were treated for 18 hours with 20 microlitres test material or vehicle, then exposed to UV light (1.7mW/cm²) or dark, for 1 hour. Cultures were incubated overnight, then a MTT cytotoxicity assay was performed, with results expressed as % control MTT conversion. The testing strategy was to assess the phototoxicity of 2 concentrations at the maximum causing no significant dark toxicity (up to 100mg/ml). The criterion for phototoxicity was a 30% difference in dark/light toxicity at one concentration. This strategy minimised the number of cultures used and therefore the cost of testing. EpiDerm™ was also compared with SkinEthic™ (Laboratoire SkinEthic™, Nice, France) using light microscopy and by assessing the phototoxicity of anthracene. The EpiDerm™ assay correctly identified the known phototoxicity of the chemicals tested. The ingredients were found to be non-phototoxic, confirming previous 3T3 assay results but at more relevant concentrations. The results also indicated that both culture models could be used to detect the phototoxic hazard of anthracene and by inference other materials. However, further investigations may be needed to determine if the same order of potency of materials is given by the models compared with human *in vivo* data.

EFFECTS OF CHEMICALLY MODIFIED TETRACYCLINES ON MATRIX METALLOPROTEASEAND PSA IN PROSTATE TUMOR CELL LINES

M. Kothari, S.R. Simon, Dept of Pathology & Dept of Biochemistry and Cell Biology, SUNY Stony Brook, NY 11794

The effect of derivatives of Chemically Modified Tetracycline-300 (6-demethyl, 6-deoxy, 4dedimethylamino tetracycline, CMT-300) on expression of Matrix Metalloprotease-9 (MMP-9) by the PC3 cell line was examined using gelatin zymography and ELISA. We also examined the effect of these derivatives on the levels of Prostate Specific Antigen (PSA) produced by the LNCaP cell line using ELISA. PC-3 is an androgen-unresponsive cell line that may be viewed as a model for advanced prostate cancer. TGF-βl at concentrations of 0.1 to 10 ng/ml markedly augmented MMP-9 production by PC-3 cells in a dose-dependent manner. CMT-308, a ring-substituted derivative of CMT-300, attenuated this growth factor-induced increase in MMP-9 secretion. In gelatin zymograms, higher molecular weight aggregate forms of MMP-9 were prominent in the presence of TGF-β alone and were significantly diminished in the presence of growth factor and CMT-308. The decrease in total MMP-9 levels was confirmed using an ELISA that detects pro-, active and TIMP-bound forms of MMP-9. On the other hand, CMT-308 did not reduce levels of the serine protease tumor marker PSA secreted by LNCaP, an androgen-sensitive cell line which may be considered as a model of early prostate cancer. PSA levels were determined by an ELISA for the free protease. By contrast, PSA levels produced by LNCaP cells were decreased by ~84% upon treatment with CMT-300, and by ~76% upon treatment with CMT-306, another substituted derivative of CMT-300. Levels of PSA released were normalized for cell number using a DNA-binding fluorescence assay for total cell number. The diminution in normalized PSA levels was correlated with preferential cytotoxicity of the different CMTs towards LNCaP cells as compared with normal prostate stromal and epithelial cells. Our findings suggest that the ability of CMT300 and its derivatives to down-regulate MMP-9 levels may be of use in therapy of prostate cancers with high metastatic potential. Modulation of PSA levels may be a further indication of the capacity of the CMTs to attenuate the invasive and metastatic phenotype of prostate tumor cells. These observations support further development of the CMTs in management of prostate cancer. [Supported by USAMRMC (DAMD17-98-1-8560), NIDCR (R0l-DE-10985), NYS Office of Science and Technology (SUSB Center for Biotechnology), and CollaGenex Pharmaceuticals, Inc.]

Notes:

MYC NETWORK GENE EXPRESSION IN DEVELOPING NORMAL MURINE PROSTATE AND IN PROSTATIC CELL LINES IN RESPONSE TO THE SOY ISOFLAVONE GENISTEIN.

Leo Kretzner, Barry G. Timms, Qi Luo[1], Erin Harmon[2] , and Scott Keckler. Basic Biomedical Sciences Division; University of South Dakota School of Medicine; Vermillion, SD 57069, USA ([1]The Wistar Institute, Philadelphia, PA; [2]Developmental Biology Program, Stanford University, Stanford, CA) [kretzner@usd.edu; btimms@usd.edu]

c-Myc expression in normal developing prostate is well established, as is its overexpression in many prostatic tumor samples. Numerous genes have been discovered in the past decade, however, related to c-myc but antagonizing its effects: the Mad and Mxi genes are typically expressed upon cell differentiation, rather than in cycling cells. The expression of these, as well as the c-myc homologues Land N-myc, has been completely uncharacterized in normal or cancerous prostate growth. This is of particular relevance because Mxi1 is lost or mutated in some human prostate tumors, and mouse Mxi1null mutants exhibit prostatic hyperplasia.

We have characterized the expression patterns of these genes during normal murine prostate development, and find they show novel patterns of expression in prostate gland compared to other systems studied. Of particular interest, Mad1 and Mad4, markers of late differentiative stages in other tissue types, were continually expressed during both proliferative and differentiative phases of prostate development. We also find previously unreported strong expression of L-myc throughout murine prostate development, while that of Mxi1 was modest and confined to middle and late developmeiital stages. Intriguingly, Mad1 is also present in proliferating normal prostate cell cultures but not in CaP tumor cell lines, suggesting Mad1 expression may be clinically relevant. Mad1-null mice are now being studied. We are also beginning to characterize the effects of the weakly estrogenic soy isoflavone genistein on gene expression in both normal primary prostate epithelial cells and CaP tumor cell lines. Effects of genistein on multiple genes including but not limited to the Myc Network are being studied. An indication that Myc expression may play a role in genistem cytostatic effects, however, comes from a system of isogenic c-myc+/+ and c-myc-/- fibroblasts: Low to modest concentrations of genistein (e.g., 10 µg/ml) which are dramatically cytotoxic to myc+ cells have minimal effects on the myc-null derivatives.

Notes:

EXPRESSION OF VITAMIN D RECEPTOR IN HUMAN PROSTATE

Krill, D., Biscegha, M., DeFlavia, P., Luo, J., Dhir, R., Becich, M.J., Leman, E., and Getzenberg, R.H., University of Pittsburgh School of Medicine, Pittsburgh USA

UV exposure and serum levels of vitamin D have been linked in several studies with prostate cancer risk. In the cell the action of vitamin D is mediated through vitamin D receptors (VDR). We examined the presence of VDR in normal human prostate tissue from donors of various ages to determine if the VDR expression pattern changes with increasing age.

Donors were identified from the Western Pennsylvania Tissue Bank which were obtained between the years 1994 and 2000. Immunohistochemical studies were performed on paraffin-embedded tissue from the following age decades: 10-19, 20-29, 30-39, 40-49, 50-59, 60-69. A statistical comparison of the VDR expression was performed by age of the donor and location in the prostate. The presence of VDR expression in prostate was compared with expression in different types of human tissues.

Mean VDR expression was lowest in the 10-19 age group (1.875+/-.315). The intensity of the nuclear VDR increased with age through the fifth decade (2.583+/- .271), and then dropped to a mean of 2.00+/-.577 for cases of age 60-70. The drop in VDR expression between the fifth and sixth decade was 22%. Though there are no previous studies in human prostate, these findings confirm studies in rats that showed 22% less VDR protein with age. When multiple sections of the same donor prostate were compared, VDR expression markedly declined in the direction of the central zone compared to the peripheral zone.

Notes:

Inhibition of Tumor Cell Invasiveness by Chemically Modified Tetracyclines

Gu Y.*[1], Lee H.M.[3], Roemer E.J.[2], Musacchia L.[1], Golub L.M.[3] and Simon S.R.[1,2]

Departments of Biochemistry & Cell Biology[1], Pathology[2], and Oral Biology & Pathology[3], SUNY at Stony Brook, Stony Brook, NY 11794, USA

Abstract: COLO 205 is a cell line derived from a human colon carcinoma with high degradative activity towards extracellular matrix (ECM). It has been shown that COLO 205 cells produce matrix metalloproteinases (MMPs). MMPs are a family of enzymes known to degrade components of the ECM and have been implicated in tumor invasion. In the present study, we have analyzed the multiple effects of chemically modified tetracyclines (CMTs) on the expression and activity of MMPs secreted by COLO 205 cells *in vitro* with the aim of evaluating these compounds for potential use in management of invasive tumors. Because COLO 205 cells can degrade an interstitial ECM in serum-free medium *in vitro*, we have been able to compare the effects of the tetracyclines on this measure of invasive activity with their effects on proteinase expression and activity. We demonstrate here that one of the chemically modified tetracyclines, 6-deoxy-6-demethyl-4-de(dimethylamino)tetracycline (CMT-3) can effectively inhibit ECM degradation mediated by COLO 205 cells or their conditioned medium. Gelatin zymography and immunoblots show that CMT-3 has the ability to inhibit release of MMP-2 into conditioned medium as well as to inhibit MMP-2 gelatinolytic activity, which correlates with the results from ECM degradation assays. On the basis of our findings with COLO 205 cells we have expanded our evaluation of the tetracyclines to include effects on a genetically engineered line of MDA-MB-231 breast tumor cells overexpressing MMP-9 at levels over tenfold those of the parent cell line, and on three human prostate tumor cell lines, LNCaP, DU-145, and PC-3. We show here that CMT-3 displays multiple modes of action: inhibiting MMP activity, reducing levels of MMP expression, and exhibiting selective cytotoxicity towards some of the tumor cell lines.

INTRODUCTION

The Matrix Metalloproteinases (MMPs) constitute a family of zinc-dependent enzymes known to degrade components of the extracellular matrix (ECM). In view of their ability to degrade basement membrane as well as interstitial stromal ECM components, MMPs have been implicated in tumor cell invasion and metastasis [1]. There are numerous examples of expression of one or more members of the MMP family *in vivo* at sites of invasive malignancy or *in vitro* in cultures of tumor-derived cell lines [2]. COLO 205 is a human colon cancer cell line which shows significant *in vitro* degradative activity towards matrix components even when plasma proteinases or viable stromal cells are not present to assist in degradation [3]. In this respect, COLO 205 differs from other tumor cell lines which can be shown to secrete MMPs, but which fail to convert the proteinases from their inactive precursors to active species in the absence of plasma proteinase- or stromal cell-mediated activation. In the course of screening for new tumor markers, it was discovered that COLO 205 cells secrete a serine proteinase with trypsin-like properties, designated tumor-associated trypsin (TAT) [4]. This serine proteinase has been shown to be capable of activating MMPs, and could contribute to an endogenous activation pathway which COLO 205 cells could employ [5]. We have analyzed the multiple effects of doxycycline and chemically modified tetracyclines on the expression and activity of MMPs and TAT secreted by COLO 205 cells *in vitro* with the aim of evaluating these compounds for potential use in management of invasive tumors. Because COLO 205 cells can degrade an interstitial ECM in serum-free medium *in vitro*, we have been able to compare the effects of the tetracyclines on this measure of invasive activity with their effects on proteinase expression and activity. On the basis of our findings with COLO 205 cells we have expanded our evaluation of the tetracyclines to include effects on a genetically engineered line of MDA- MB-231 breast tumor cells overexpressing MMP-9 at levels over tenfold those of the parent cell line, and on three human prostate tumor cell lines, LNCaP, DU-145, and PC-3. We show here that one of the chemically modified tetracyclines, 6-deoxy-6-demethyl-4-de(dimethylamino)tetracycline (CMT-3) displays multiple modes of action: inhibiting MMP activity, reducing levels of MMP expression, and exhibiting selective cytotoxicity towards some of the tumor cell lines.

MATERIALS AND METHODS

Samples of doxycycline and a series of chemically modified tetracyclines were generously provided by

*Address correspondence to this author at the Departments of Biochemistry & Cell Biology, SUNY at Stony Brook, Stony Brook, NY 11794, USA

0929-8673/01 $28.00+.00

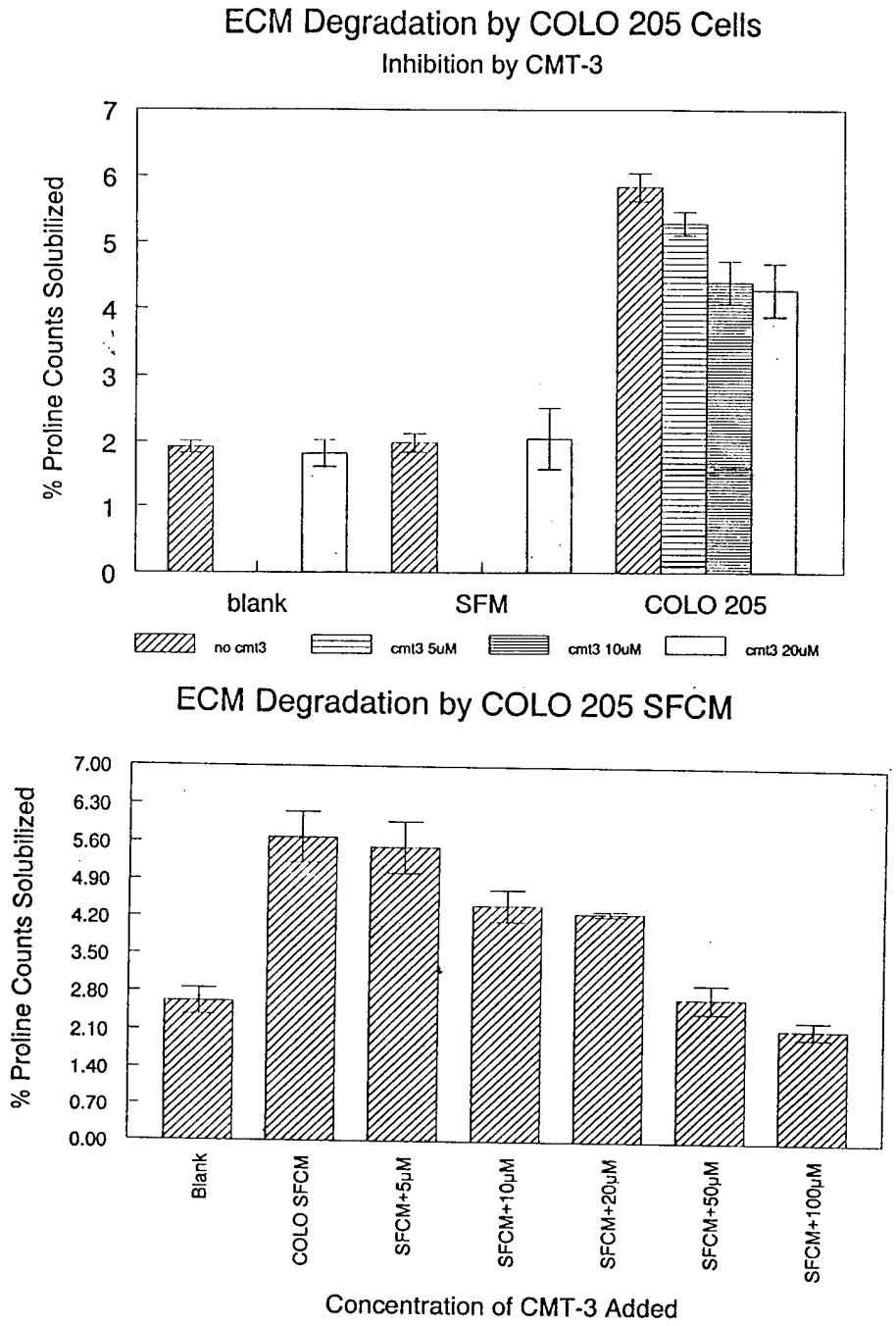


Fig. (1). a. Solubilization of R22 ECM by COLO 205 cells. Cells were plated onto wells at an initial density of 2.5 x 10^4 cells/cm^2 in serum-free RPMI 1640 medium (SFM) containing penicillin and streptomycin along with different concentrations of CMT-3 for 48 hours. Control wells contained medium only. Supernatant medium was collected for measurement of solubilized ^{3}H-proline, and the remaining ECM was solubilized with 2 M NaOH to determine percent of total counts which were solubilized by the tumor cells. b. Solubilization of R22 ECM by conditioned serum-free medium obtained from 24 hour- old cultures of COLO 205 cells. The medium was applied directly to wells along with different concentrations of CMT-3 and incubated for 2 days before collection for measurement of solubilized ^{3}H-proline and subsequent solubilization of residual ECM with 2 M NaOH as described.

CollaGenex Pharmaceuticals, Inc. The cell lines COLO 205, LNCaP, DU-145, and PC-3 were all obtained from the American Type Culture Collection. The E-10 line of MDA-MB-231 cells overexpressing MMP-9 was obtained from Dr. N. Ramos-DeSimone in the laboratory of Dr. J.P. Quigley at Stony Brook. We have maintained multiple passages of R22 rat heat smooth muscle cells which we use to elaborate a complete interstitial ECM for measurement of tumor cell-mediated degradative activity as originally described by Jones and DeClerck [6]. Normal human prostate stromal fibroblasts were obtained from Clonetics Corp. for elaboration of a prostate interstitial ECM. Our preparation of ECM from R22 cells has been described previously [7]. Briefly, the cells were cultured in multiwell microplates

until confluent, at which time the medium was supplemented with ascorbate or Mg-ascorbyl phosphate and radiolabled precursors for preferential labeling of ECM components. [^{3}H]-proline and [^{35}S]-sulfate were employed to preferentially label collagen and proteoglycans, while [^{3}H]-fucose and [^{35}S]-cysteine/methionine were employed to preferentially label adhesion glycoproteins and other noncollagenous proteins. Culture was continued for an additional 1-2 weeks with regular changes of labeled medium, at which time the cells were lysed with 25 nM NH_4OH, leaving an insoluble ECM bound to the wells of the microplates. We have shown by sequential enzymatic digestion that the R22 interstitial ECM contains about 37% collagen (predominantly type I with some type III), 11% elastin, and the balance a mix of trypsin- sensitive adhesion glycoproteins and proteoglycans. A similar procedure was employed to prepare an ECM from normal prostate stromal fibroblasts.

Tumor cells were cultured on ECM-coated wells for two days to measure degradative activity, which was quantitated as the fraction of the total ECM labels solubilized by the

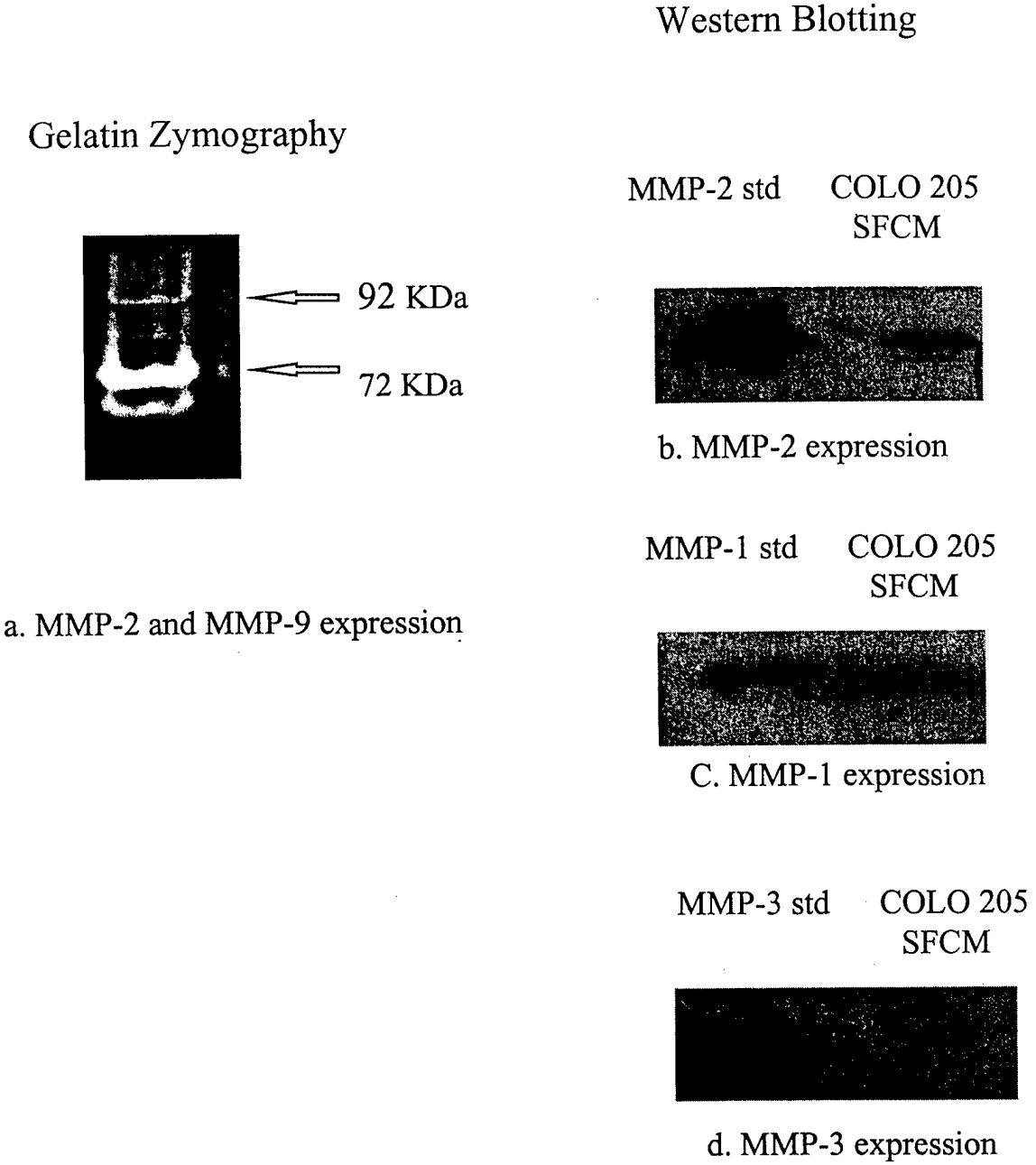


Fig. (2). a. Gelatin zymogram of serum-free conditioned medium (SFCM) obtained from 24 hour-old cultures of COLO 205 cells. Gelatinolytic zones corresponding to species derived from gelatinase A (MMP-2, 72 kDa) and gelatinase B (MMP-9, 92 kDa) were visualized after separation by SDS-PAGE. b. Western blot of (SFCM) from COLO 205 cultures along with an MMP-2 standard after separation by SDS-PAGE and probing with a mAb to MMP-2. c. COLO 205 SFCM was separated by SDS-PAGE along with an MMP-1 standard and the Western blot was probed with a mAb to MMP-1. d. COLO 205 SFCM was separated by SDS-PAGE along with an MMP-3 standard and the Western blot was probed with a mAb to MMP-3.

cells. Residual ECM was solubilized with 2 N NaOH to obtain a measure of total ECM counts. In separate experiments, conditioned medium was obtained from tumor cells cultured on unlabeled ECM or on plastic for 24 hours in serum-free medium and was applied directly to the ECM-wells to measure the degradative activity of the proteinases released by the cells during culture. Doxycycline and CMTs were dissolved in dimethylsulfoxide and added to cells either prior to their application to radiolabeled ECM or at the beginning of the degradative assays.

Cells cultured on interstitial ECM were regularly examined by light microscopy for morphological changes, including such signs of cytotoxicity as membrane blebbing and lysis. Cytotoxicity was quantitated by measuring the absorbance of the reduced formazan products of two tetrazolium salts, MTT and MTS [8]; by recording the fluorescence of the product generated by reduction of the dye Alamar Blue [9]; and by using a chromogenic coupled enzyme assay for lactic dehydrogenase released into the supernatant medium.

Proteinases released into the culture medium were detected by SDS-PAGE on gelatin or casein-impregnated gels (Novex), followed by removal of SDS and partial renaturation for zymographic analysis. Zones of enzymatic activity appear as clear bands against a Coomassie blue-stained background. More definitive identification of proteinases and inhibitors was made by electrotransfer from SDS-PAGE to PDVF membranes followed by immunoblotting with various specific monoclonal antibodies.

RESULTS

1. Tetracyclines Inhibit ECM Degradation by Tumor Cell Lines and Their Conditioned Medium

COLO 205 cells solubilized ~6% of the [^{3}H]-proline counts incorporated into the R22 ECM over the time course of 48 h when cultured in serum-free medium. This degradation could be inhibited by CMT-3: in the presence of 10 μM or 20 μM CMT-3, solubilization of [^{3}H]- proline counts was inhibited by ~30-40% (Fig. (**1a**)). Doxycycline had only a modest effect on COLO 205-mediated ECM degradation. Conditioned medium from COLO 205 cells cultured in serum-free conditions also solubilized the ECM, and this degradation could be inhibited by CMT-3 in a dose-dependent fashion. Higher concentrations of CMT-3 (50

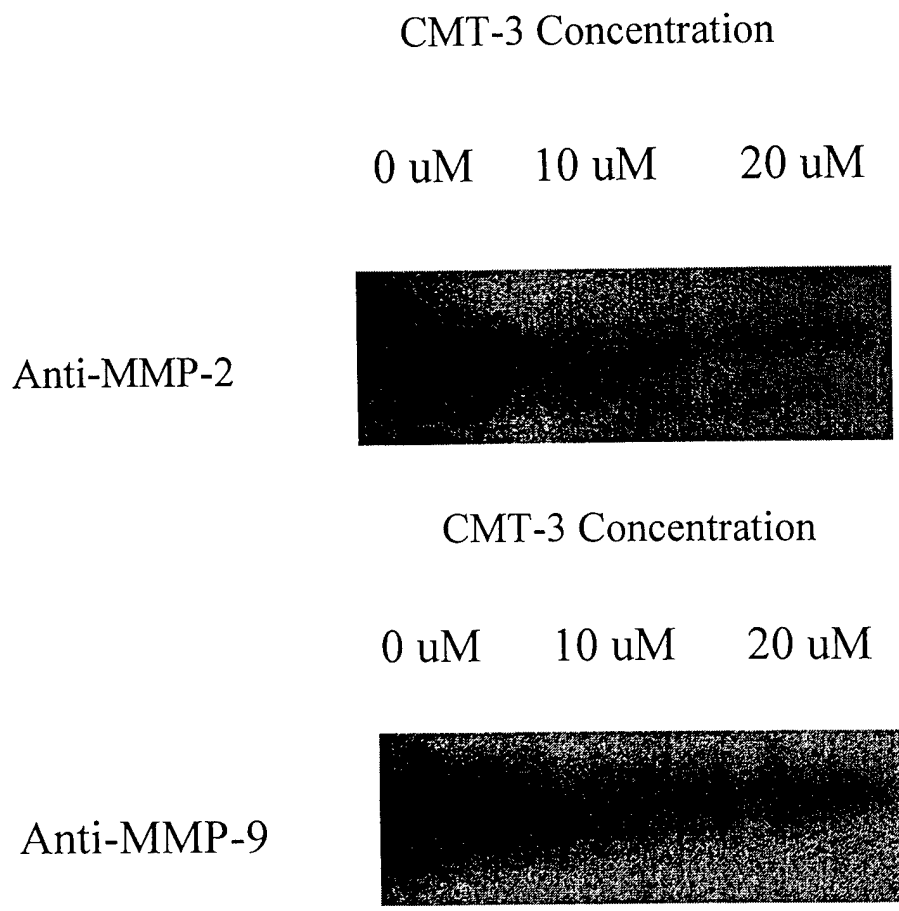


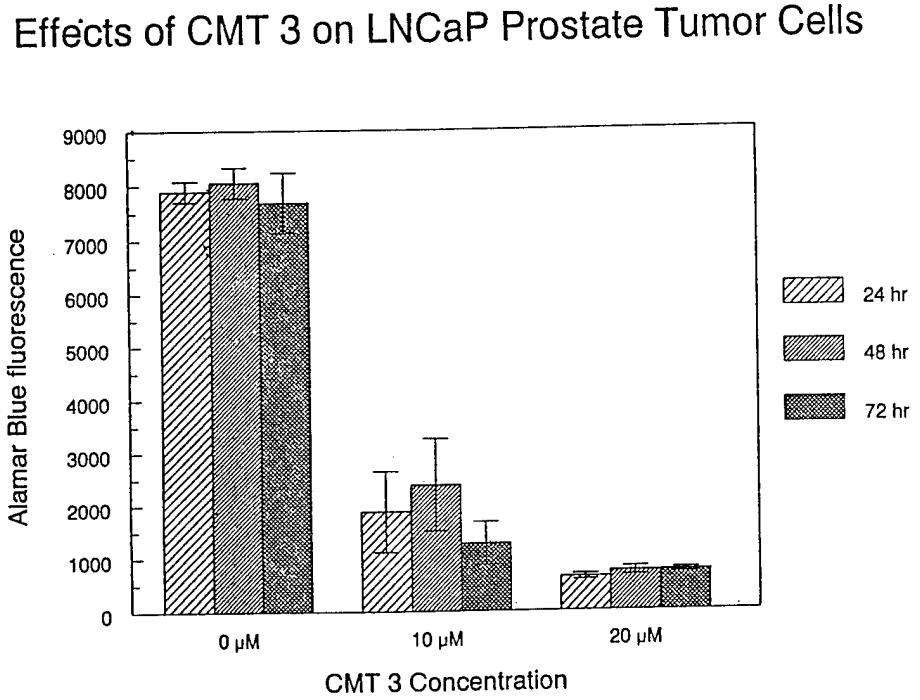
Fig. (3). a. COLO 205 cells were cultured for 24 hours in SFM in the presence of different concentrations of CMT-3. The SFCM was separated by SDS-PAGE and the Western blot was probed with a mAb to MMP-2. b. The E-10 transfected subclone of MDA-MB-231 cells was cultured for 48 hours in serum-free DMEM medium (SFM) supplemented with nonessential amino acids and pyruvate in the presence of different concentrations of CMT-3. The SFCM was separated by SDS-PAGE and the Western blot was probed with a mAb to MMP-9.

μM and 100 μM) reduced solubilization of counts by the serum-free conditioned medium (SFCM) virtually to baseline levels (Fig. (**1b**)). Significant inhibition of SFCM-mediated matrix solubilization could also be achieved in the presence of 10 mM 1,10-phenanthroline, a known inhibitor of MMPs. However, no significant inhibition of ECM solubilization by the SFCM could be achieved in the presence of CMT-5, a pyrazole derivative of tetracycline in which the oxo moieties are blocked and which shows no inhibitory activity towards purified MMPs in peptidolytic assays.

2. COLO 205 Cells Release MMPs, TIMPs, and a Trypsin-like Serine Proteinase

Analysis of the serum-free conditioned medium (SFCM) from cultures of COLO 205 cells by gelatin zymography revealed the presence of lytic zones assigned to 92 kDa and 72 kDa gelatinases; the 72 kDa species appeared as the more abundant of the two species. These MMPs in the SFCM were detected not only as major clear zones at molecular weights consistent with those of the precursor forms, but also as distinct lytic zones with molecular weights

4a



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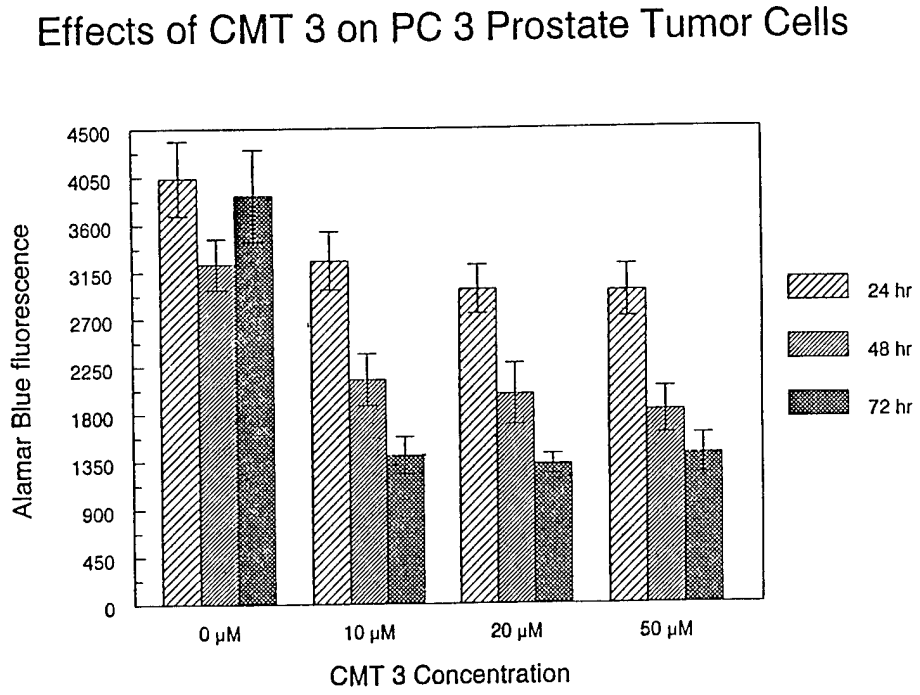

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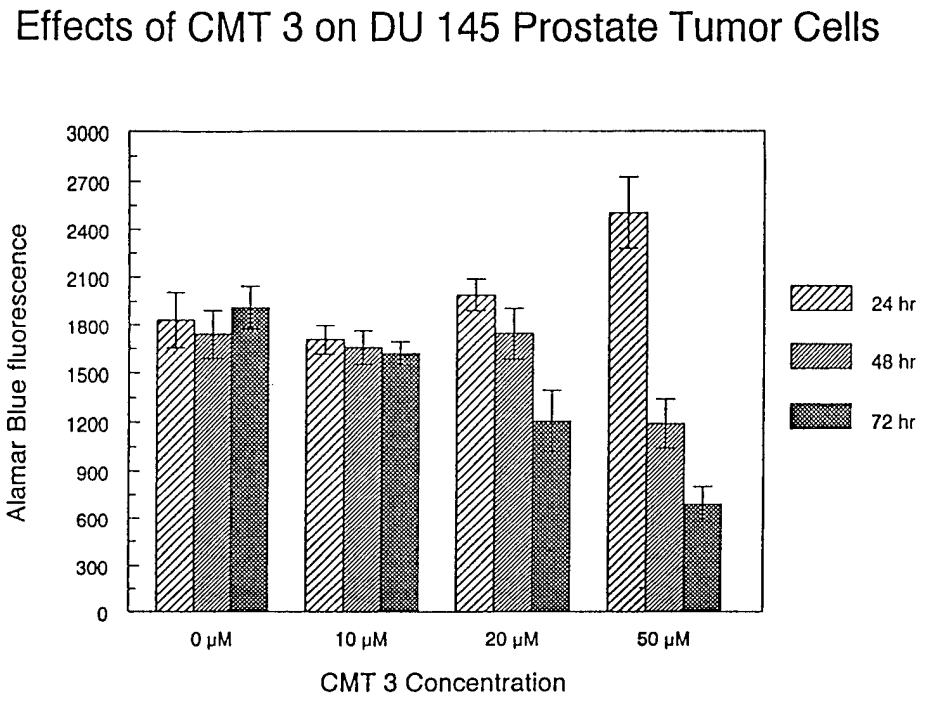

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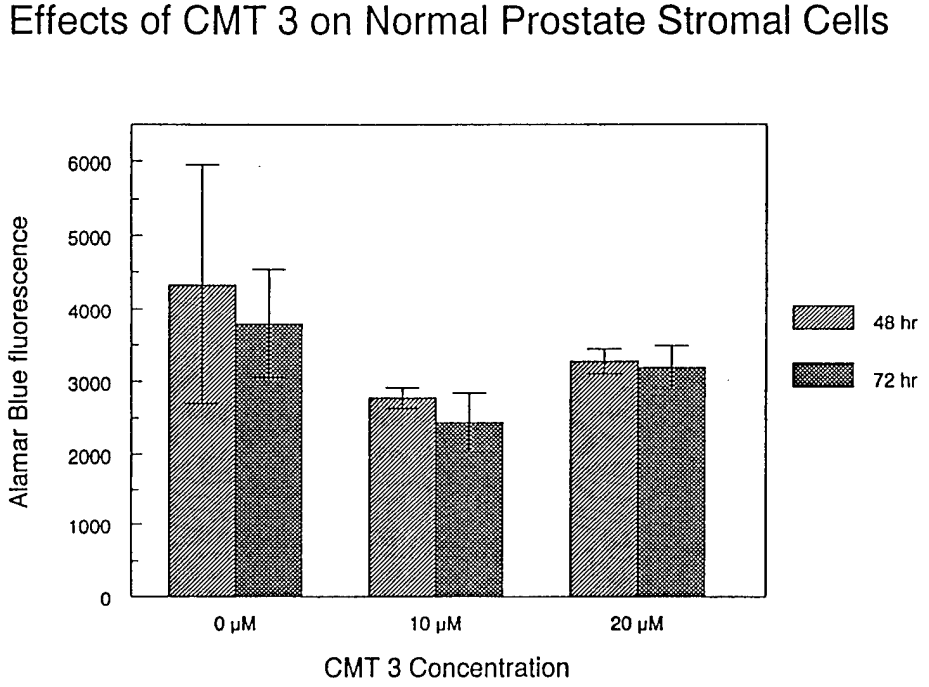

Fig. (4). a. The LNCaP line of human prostate tumor cells was cultured in the presence of different concentrations of CMT-3 and after 1, 2, or 3 days the medium was replaced with fresh medium containing the redox dye Alamar Blue. Fluorescence at 590 nm with excitation at 530 nm was determined after an additional 24 hours of incubation. b. The PC-3 line of human prostate tumor cells was cultured in the presence of different concentrations of CMT-3 and after 1, 2, or 3 days the medium was replaced with fresh medium containing Alamar Blue. Fluorescence was determined as described. c. The DU-145 line of human prostate tumor cells was cultured in the presence of different concentrations of CMT-3 and after 1, 2, or 3 days the medium was replaced with fresh medium containing Alamar Blue. Fluorescence was determined as described. d. Primary cultures of normal human prostate stromal cells were maintained in the presence of different concentrations of CMT-3 for 2 or 3 days; the medium was replaced with fresh medium containing Alamar Blue and fluorescence was determined as described.

consistent with activated species (Fig. (**2a & 2b**)). In solution, the spontaneous gelatinolytic activity of COLO 205 SFCM was very low, but this may reflect the presence of secreted MMP inhibitors in the medium. Immunoblots were positive for TIMP-1 and TIMP-2, the latter of which appeared to be the predominant species. When the TIMPs in

COLO 205 SFCM were inactivated by treatment with the reducing agent dithiothreitol followed by alkylation with iodoacetamide, soluble [^{3}H]-gelatin was converted to trichloroacetic acid-soluble radiolabeled fragments. This gelatinolytic activity is consistent with the hypothesis that at least some of the lower molecular weight zones on the zymograms reflect the presence of gelatinases in the COLO 205 SFCM which have proteolytic activity in the absence of exogenous activating agents.

Consistent with the capacity of cultured COLO 205 cells and their SFCM to solubilize [^{3}H]-proline-labeled R22 ECM is the presence of immunoreactive MMP-1 which could be visualized on Western blots (Fig. (**2c**)). Variable levels of immunoreactive MMP-3 could also be visualized by Western blotting (Fig. (**2d**)), and a -50 kDa zone could be visualized on casein zymograms of COLO 205 SFCM, but it is difficult to assign this caseinolytic activity unambiguously to an active form of MMP-1 or MMP-3. A

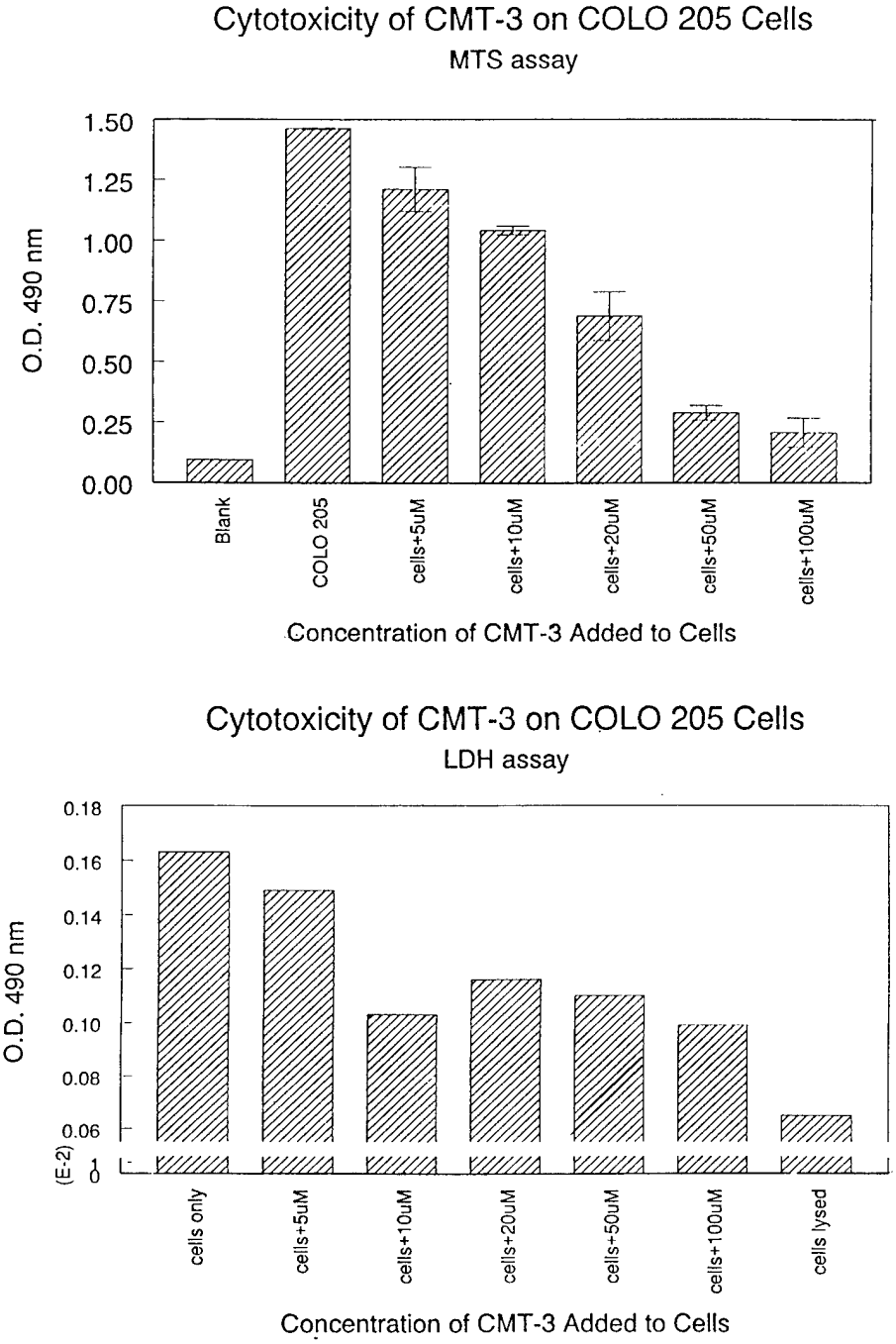

Fig. (5). COLO cells were plated onto ECM-coated wells in the presence of different concentrations of CMT-3 as described in Methods section. At the end of a 2-day incubation, a. the cells were incubated with MTS/PMS for 3-4 hours; 10% SDS was added to each well to stop the reaction. The levels of solubilized formazan were quantitated by measurement of absorbance at 490 nm. b. COLO cells were incubated with NADH/pyruvate substrate for 30 minutes at 37^{0}C, 2,4-dinitrophenyl hydrazine reagent (Sigma) was then added to each well for 20 minutes incubation at room temperature, followed by sodium hydroxide solution, and the plate was read at 490 nm.

prominent band of lysis at -29 kDa was also seen on the casein zymograms consistent with the known position for the serine proteinase TAT-2; this band could be completely ablated by incubation of the casein zymogram with soybean trypsin inhibitor during development, but was not affected by treatment with 1,10- phenanthroline or CMT-3. In contrast, development of casein or gelatin zymograms of COLO 205 SFCM in the presence of 100 μM CMT-3 ablated formation of clear zones associated with the putative MMPs.

3. Levels of MMPs are Diminished After Culture of Tumor Cells in the Presence of CMT-3

Densitometric scans of gelatin zymograms and Western blots for different MMPs in the SFCM from tumor cell lines cultured in the presence of CMT-3 showed diminished levels of some, but not all, of these proteinases. Immunoreactive MMP-1 levels (as assayed by Western blots) in COLO 205 SFCM were unaffected when the cells were cultured in the presence of 10 μM or 20 μM CMT-3 for 24 h. However, levels of immunoreactive MMP-2 (as assayed by Western blots) in the SFCM were diminished by ~10% after 24 h of culture of COLO 205 cells in the presence of 10 μM CMT-3 and by ~65% after culture in the presence of 20 μM CMT-3 (Fig. (**3a**)). Because CMT-3 has some cytotoxicity to COLO 205 cells (see below), the effects of this tetracycline on levels of MMPs released by the E-10 subclone of the MDA-MB-231 cell line, which releases high levels of MMP-9, were also measured. CMT-3 is not cytotoxic to MDA-MB-231 cells at concentrations up to 50 μM. However, levels of immunoreactive MMP-9 in the SFCM were diminished by ~45% after 24 h of culture of the E-10 cells in the presence of 10 μM CMT-3 and by ~60% after 24 h of culture in the presence of 20 μM CMT-3 (Fig. (**3b**)). Culture of COLO 205 cells or E-10 cells for 24 h in the presence of 20 μM CMT-5, the tetracycline pyrazole derivative, resulted in no diminution of levels of MMPs in the SFCM.

4. Tumor Cell Lines Show Differential Sensitivity to the Cytotoxic Effects of CMT-3

In the course of observing some tumor cell lines cultured in the presence of CMTs or doxycycline, some morphologic changes could be seen which were indicative of cytotoxicity, including vacuolization, plasma membrane blebbing, and, eventually failure to exclude Trypan blue. A more systematic study of cytotoxic effects of CMT-3 revealed some striking differential sensitivity of tumor cell lines, based on loss of capacity to reduce Alamar Blue. The most sensitive cell line examined was the human prostate tumor cell line LNCaP: marked cytotoxicity was observed after 24 h of incubation with 10 μM or 20 μM CMT-3, and virtually no cells remained viable after 48 h of exposure (Fig. (**4a**)). The prostate tumor cell line PC-3 was less sensitive to CMT-3: after 48 h of exposure to CMT-3, about half the cells had survived, but after 72 h, only about one third of the cells were still viable (Fig. (**4b**)). DU-145 prostate tumor cells were quite resistant to CMT-3: minimal loss of viability occurred after 48 h of exposure to concentrations of CMT-3 as high as 20 μM, and only about one third of the cells were nonviable after 72 h of exposure (Fig. (**4c**)). Under these conditions, there was no significant loss of viability of confluent cultures of normal prostate stromal fibroblasts (Fig. (**4d**)) or of R22 rat smooth muscle cells over a 72 h period in the presence of CMT-3. COLO 205 cells displayed intermediate sensitivity to CMT-3: after 48 h of exposure to 20 μM CMT-3, ~37% of the cells were nonviable. At higher concentrations of CMT-3, COLO 205 survival was more limited: after 48 h of exposure to 50 μM CMT-3, only 15% of the cells remained viable. Estimates of survival based on reduction of Alamar Blue, conversion of two tetrazolium salts to their formazans, and measurement of release of lactic dehydrogenase into the supernatant medium were all consistent (Fig. (**5a & 5b**)). In contrast, the E-10 subclone of MDA-MB-231 was very resistant to CMT-3, with 92% survival after 48 h of exposure to 20 μM CMT-3 and 82% survival after 48 h exposure to 50 μM CMT-3 (Fig. (**6a & 6b**)). Neither COLO 205 cells nor MDA-MB-231 cells showed any cytotoxicity in the presence of doses as high as 100 μM of CMT-5, the tetracycline pyrazole derivative.

DISCUSSION

The results presented here are consistent with the conclusion that 6-deoxy-6-demethyl-4-de(dimethylamino)methyltetracycline (CMT-3) has multiple effects on tumor cells, including direct inhibition of MMP proteolytic activity, diminution of MMP levels in the surrounding medium, and cytotoxicity towards some, but not all, tumor cell lines. These effects could all be advantageous in controlling the invasive and metastatic spread of tumors *in vivo*. The mechanisms of these multiple effects are not all consistent with the capacity of the CMTs to bind divalent cations such as the essential zinc ions in MMPs. For example, CMT-5, which lacks metal binding activity, has been shown to diminish levels of MMPs in the surrounding medium of various cells in culture, and has recently been reported to diminish levels of the serine proteinase TAT-2 in the surrounding medium of COLO-205 cells, a property which it shares with CMT-3 [T. Sorsa, personal communication]. The mechanism of differential cytotoxicity of CMT-3 towards tumor cell lines remains elusive. It is true that more highly proliferative cultures of cell lines are more sensitive to CMT-3, suggesting that the tetracycline derivative has differential effects at different points in the cell cycle, but it is also true that some neoplastic cell lines, like DU-145 and MDA-MB-231, which are proliferating in culture, are as resistant to CMT-3 as confluent normal stromal cells, which have markedly diminished proliferative activity. Efforts are currently underway to elucidate the molecular mechanisms of the differential cytotoxic effects of CMT-3 so that new chemically modified tetracyclines with enhanced cytotoxicity to different tumors can be developed.

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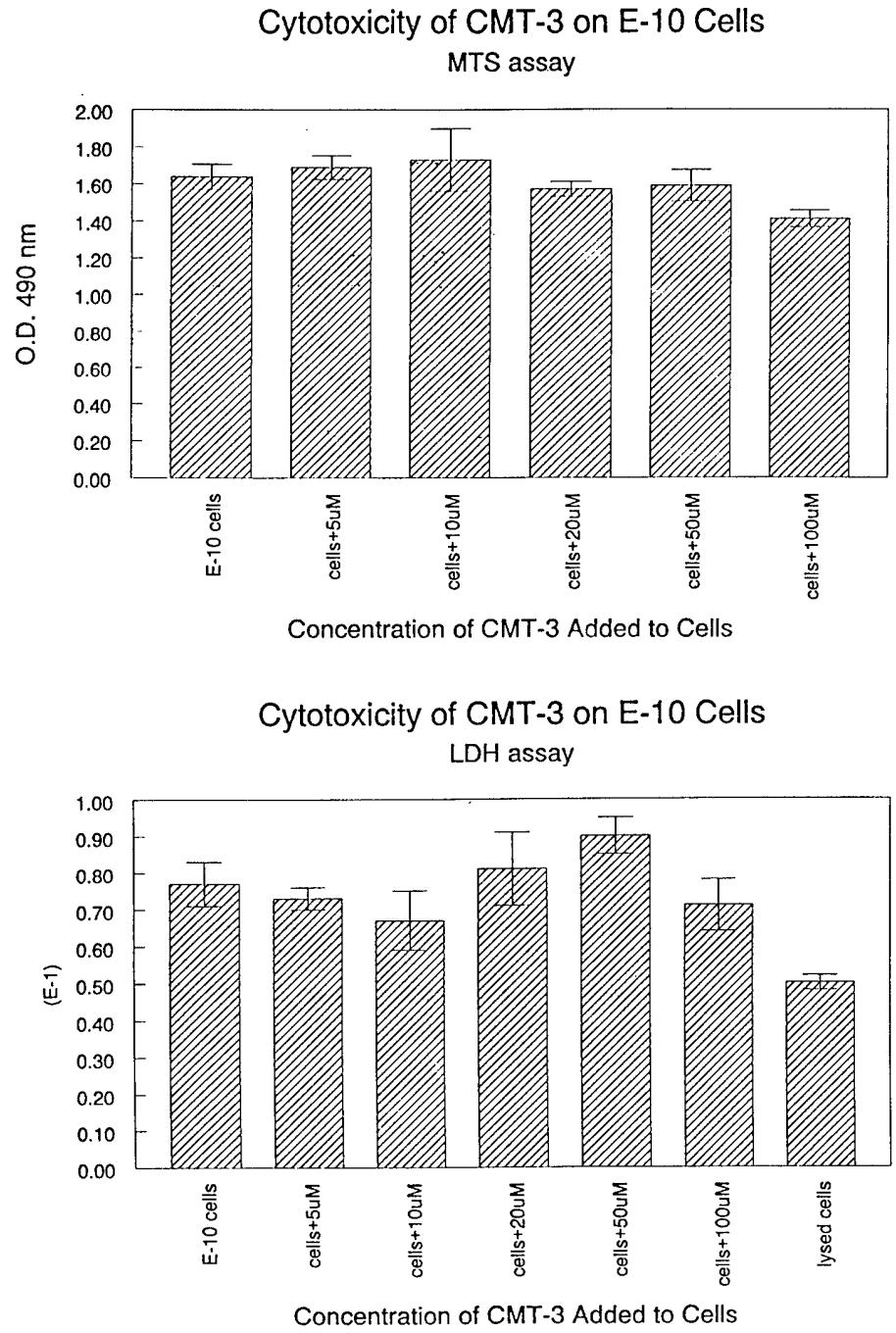

Fig. (6). E-10 cells were plated onto ECM-coated wells in the presence of different concentrations of CMT-3 as described in Methods section. At the end of a 2-day incubation, a. the cells were incubated with MTS/PMS for 3-4 hours; 10% SDS was added to each well to stop the reaction. The levels of solubilized formazan were quantitated by measurement of absorbance at 490 nm. b. the cells were incubated with NADH/pyruvate substrate for 30 minutes at 37^0C, 2,4- dinitrophenyl hydrazine reagent (Sigma) was then added to each well for 20 minutes incubation at room temperature, followed by sodium hydroxide solution, and the plate was read at 490 nm.

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Evaluation of the phototoxic potential of chemically modified tetracyclines with the 3T3 neutral red uptake phototoxicity test

Brad Zerler[1], Elizabeth Roemer[2], Hans Raabe[3], Angela Sizemore[3], Allison Reeves[3] and John Harbell[3]
[1]CollaGenex Pharmaceuticals, Newtown, PA, USA; [2]Department of Pathology, Stony Brook, NY, USA; and [3]Institute for In Vitro Sciences, Inc., Gaithersburg, MD, USA

Abstract. Chemically modified tetracyclines (CMT) lack antimicrobial activity, have the ability to inhibit matrix metalloproteases, and in some cases, serine proteases, thus attenuating connective tissue degeneration. Several of these compounds are now in development. Since the tetracyclines are known to be photo-active (and some phototoxic), an *in vitro* screening programme was begun. To identify potentially phototoxic CMTs, the 3T3 neutral red uptake phototoxicity test was employed. 3T3 cells were seeded onto 96-well plates and incubated overnight. The growth medium was removed and replaced with phenol-red free Hanks' Balanced Salt Solution containing serial dilutions of the CMTs (two plates per compound). After an initial 1 hour incubation at 37°C, one plate was exposed to 5 Joules/cm^2 of UVA/white light from a solar simulator, while the other was kept in the dark. The plates were then rinsed, re-fed and incubated for 24 hours. Cell viability was measured by neutral red uptake. Phototoxicity was measured by the relative toxicity between the doses with and without light exposure, following published guidelines. Reference compounds included commercially available tetracycline, doxycycline, and minocycline. The phototoxic response of the compounds in the assay was consistent with their behaviour *in vivo*. These phototoxicity results, combined with efficacy data, facilitate rational choices in selecting compounds for further study and development.

Introduction

Tetracyclines have been previously demonstrated to inhibit the breakdown of connective tissue by mechanisms separate from their antibiotic activities [1]. Subsequent studies have shown that a number of chemically modified tetracyclines (CMTs), lacking the antibiotic moiety, are potent enzyme inhibitors [2]. Evaluation of a panel of these CMTs is currently under way, to determine which will be the best candidates for pharmaceutical development. An overall *in vitro* testing regimen has been undertaken to rank the compounds for both efficacy and toxicity. Since the parent tetracycline compounds are known to be photo-active and in some cases phototoxic, it was deemed necessary to screen the chemically modified derivatives for their potential phototoxicity.

The 3T3 neutral red uptake phototoxicity test has undergone extensive validation under the direction of the European Union and COLIPA [3,4]. The

Address for correspondence: Dr Brad Zerler, CollaGenex Pharmaceuticals, 41 University Drive, Newtown, PA 18940, USA.

assay uses 3T3 cells treated with dilutions of the test material in the presence or absence of a non-cytotoxic dose of UVA and visible light. Phototoxicity is predicted from the relative cytotoxicity observed between the two treatments. The 3T3 neutral red uptake phototoxicity test was used to evaluate the phototoxicity of CMTs. Reference compounds, minocycline, tetracycline, and doxycycline, were used to gauge the *in vitro* responses against known activity in humans for this class of compounds. The structures of these three compounds are shown in Fig. 1.

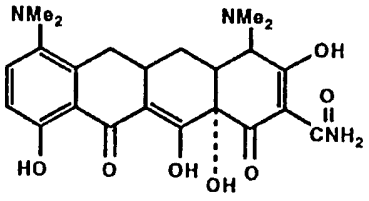
Materials and methods

Cells

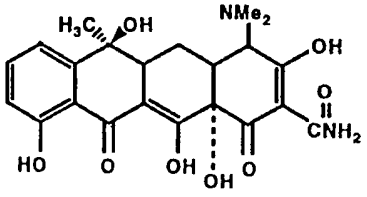
BALB/c 3T3 (CCL-163) were obtained from the ATCC (Manassas, Virginia, USA) and cultured in antibiotic-free Dulbecco's Minimum Essential Medium (4.5 g/l glucose) (DMEM; Quality Biological, Gaithersburg, MD, USA) supplemented with L-glutamine (4mM) and 10% newborn calf serum (Hyclone, Logan, Utah, USA). The working cell bank was prepared and was found to be free of mycoplasma. Stock cultures were maintained in antibiotic-free medium and passaged every other working day. Streptomycin sulphate (100 μg/ml) and penicillin (100 IU/ml) were added to the medium only after the cells were treated with the test article in 96-well plates.

Chemically modified tetracyclines

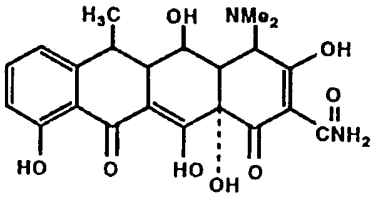
CMTs were prepared in dimethylsulfoxide (DMSO) and coded before shipment to the laboratory. Serial dilutions were prepared in DMSO at concentrations 100x the



Minocycline



Tetracycline



Doxycycline

Fig. 1. Structures of the three reference compounds.

final testing concentrations. The CMT dilutions in DMSO were then diluted in Hanks' Balanced Salt Solution (HBSS; Life Technologies, Gaithersburg, Maryland, USA) for application to the cells. HBSS was selected as the dosing buffer, since CMTs are relatively insoluble in aqueous medium and become less so as the pH becomes more alkaline. The final DMSO concentration was 1% in treated and control cultures. For the dose range-finding assay, eight serial dilutions covered a range of 100–0.03 mg/ml in half-log steps, while the definitive assays used 6–8 doses prepared in quarter-log steps, centred on the expected 50% toxicity point. In many cases, the dose range for treatment without UV light was different from the dose range selected with UV light. Dose ranges with and without UV light were made to overlap, so that calculations of the mean photo effect (MPE) could be performed. The highest dose recommended to prevent false negative results from UV absorption by the dosing solutions is 100 μg/ml.

Controls

Each assay included both negative (solvent) and positive controls. Twelve wells of negative control cultures were used on each 96-well plate. Chlorpromazine (Sigma, St Louis, MO, USA) was used as the positive control and was prepared and dosed like the CMTs.

Solar simulator

A Dermalight SOL 3 solar simulator (Dermalight Systems, Sherman Oaks, California, USA), equipped with a UVA H1 filter (320–400 nm), was adjusted to the appropriate height. Measurement of energy through the lid of a 96-well microtiter plate was carried out by using a Hönle UVA radiometer. Simulator height was adjusted to deliver 1.7 ± 0.1 mW/cm^2 of UVA energy (the resulting dose was 1 J/cm^2 per 10 minutes).

Phototoxicity assay (Fig. 2)

Duplicate plates were prepared for each test material by seeding 10^4 3T3 cells per well in 100 μl of complete medium and incubating for 24 hours before treatment (37 ± 1°C and 5 ± 1% CO_2). Only the interior wells of the plate were used. Prior to treatment, the medium was removed, and the cells were washed once with 125 μl pre-warmed HBSS. Fifty μl of prewarmed HBSS were added to each well. Fifty μl of test article dilutions were added to the appropriate wells, and the plates were returned to the incubator for one hour. Following the one hour incubation, the plates designated for the photoirritation assay were exposed (with the lid on) to 1.7 ± 0.1 mW/cm^2 UVA light for 50 ± 2 minutes at room temperature, resulting in an irradiation dose of 5 J/cm^2. Duplicate plates designated for the cytotoxicity assay were kept in the dark at room temperature for 50 ± 2 minutes. After the 50-minute exposure period, the test article dilutions were decanted from the plates, and the cells were washed once with 125 μl HBSS. One hundred μl of

medium were added to all wells, and the cells were incubated as above for 24 hours.

At least 23 hours after treatment, visual observations were performed and recorded for each treatment group. Visual observations were used as a check for the neutral red uptake endpoint. After 24 hours of incubation, the medium was

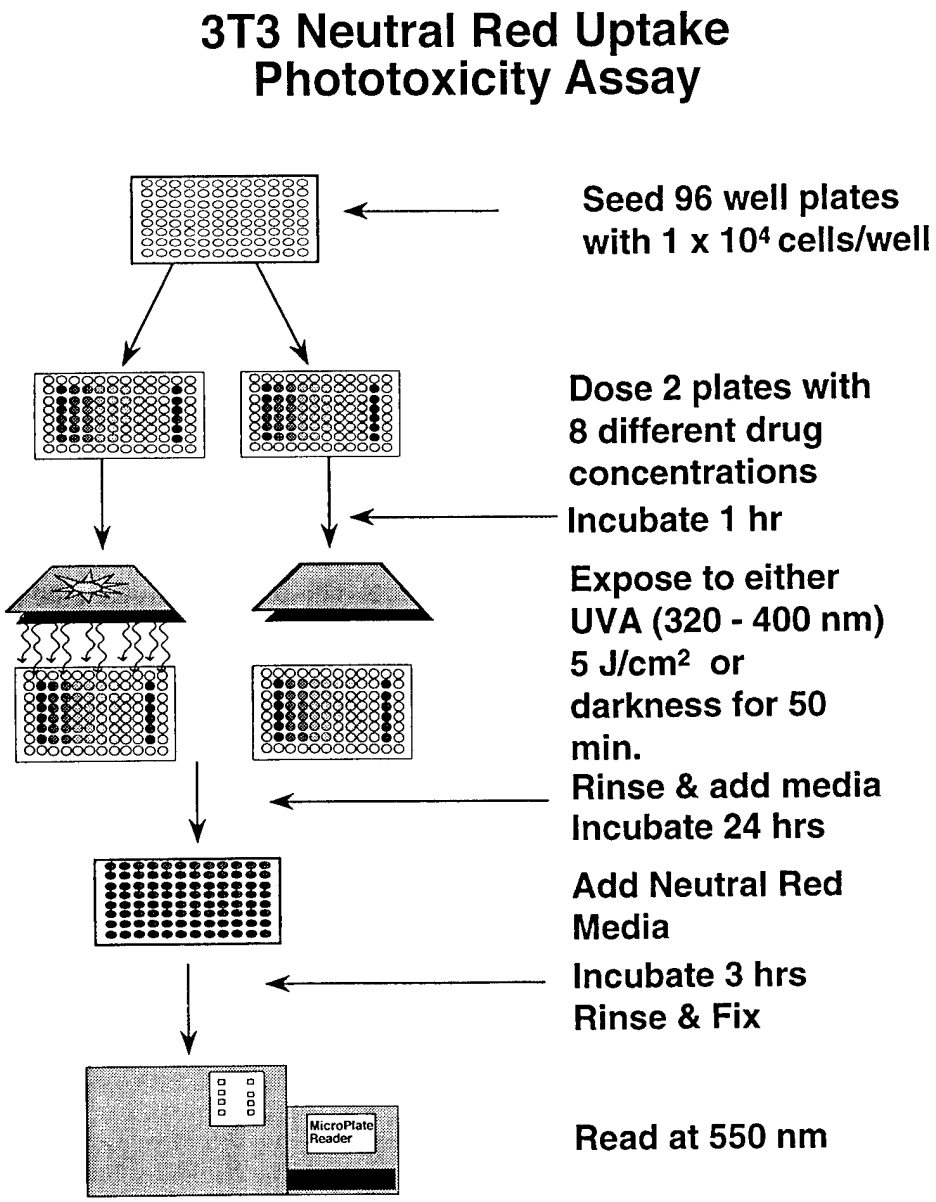


Fig. 2. Flow diagramme of the neutral red uptake phototoxicity test.

decanted and 100 μl of the neutral red-containing media added to each well. The plates were returned to the incubator and incubated for approximately 3 hours. After 3 hours, the medium was decanted and each well rinsed once with 250 μl of HBSS. The plates were blotted to remove the HBSS and 100 μl of neutral red solvent were added to each well. After a minimum of 20 minutes of incubation at room temperature (with shaking), the absorbance at 550 nm was measured with a plate reader, using the mean of the blank outer wells as the reference. Relative survival was obtained by comparing the amount of neutral red taken up by test article and positive control treated groups with the neutral red taken up by the negative group on the same plate. IC50 values for both the UVA exposed and non-exposed groups were determined whenever possible. One dose range-finding and at least two definitive trials were performed for each CMT.

Determination of phototoxicity

Two methods of analysis can be used with this assay. The first is the photoinhibition factor (PIF), which compares the ratio of the IC50(–UV)/IC50(+UV). Materials showing PIF values of 5 or greater are considered to be phototoxic (example in Fig. 3). In some cases, an IC50 cannot be determined within the acceptable dose range, particularly without UV light. In this case, a determination of the MPE is performed by using software developed under the direction of Dr H.G. Holzhütter [5]. This approach compares the two curves (–UV and +UV) over the range of active doses and measures both test article

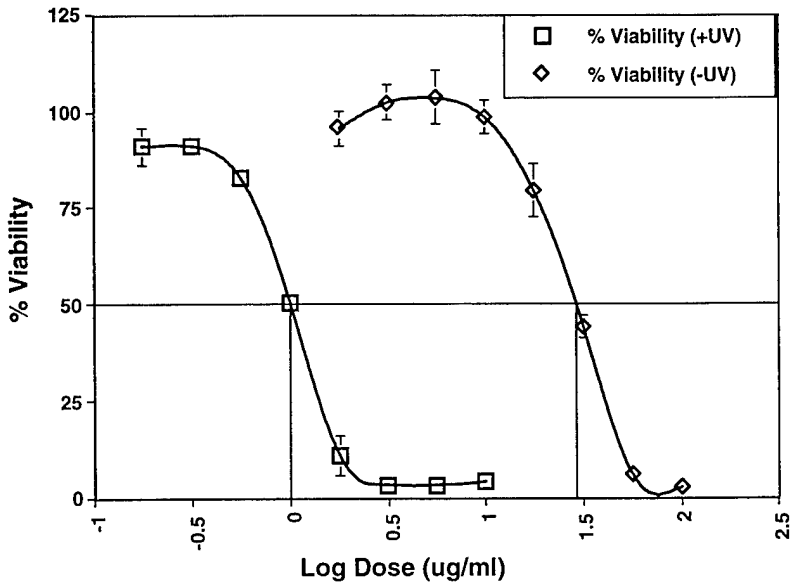


Fig. 3. Sample chlorpromazine data from a single trial. The IC50 values were 1.0 μg/ml with UVA and 29.75 μg/ml without UVA. These values produced a PIF of 29.7 and an MPE of 0.74.

concentration and UV induced changes in viability. These changes are averaged over the overlapping portions of the curves to produce the MPE. Values greater than 0.1 are considered positive, with 1 being the maximum response.

Results and discussion

Over 30 CMTs were tested in the phototoxicity assay. The CMTs were then grouped according to their phototoxic potential relative to the reference compounds, minocycline, tetracycline, and doxycycline. Phototoxic potential was judged by the MPE and the absolute IC50 of the CMT in the presence of UV light. Twelve CMTs were less toxic than the non-phototoxic benchmark, minocycline, seven fell into the weakly phototoxic range between minocycline and tetracycline, two fell between tetracycline and the phototoxic doxycycline (and chlorpromazine), and eleven were more phototoxic than doxycycline. Examples from several of these groups are shown in Table 1. Data from two definitive trials are presented for each compound.

Minocycline is generally considered to be non-phototoxic in humans [6]. In this assay, the MPEs from the first trial were weakly positive, while in the second trial

Table 1. Examples of ranking unknown CMTs against known tetracyclines based on MPE and IC50 values.

TEST ARTICLE	MPE	IC50 (μg/ml)		PIF
		+ UV	–UV	
Derivative A	0.032	>100	>100	none
MINOCYCLINE	0.339	42.9	>100	>2.3
	0.121	58.2	>100	>1.7
Derivative B	0.333	12.0	65.8	5.5
	0.615	21.2	>100	>4.9
TETRACYCLINE	0.650	19.8	>100	>5.05
	0.703	17.8	>100	>5.62
DOXYCYCLINE	0.883	4.17	98.2	23.7
	0.794	3.24	74.9	23.0
CHLORPROMAZINE	0.861	0.74	18.1	24.8
	0.915	0.74	30.8	40.9
Derivative D	0.845	0.499	>100	>208
	0.691	0.326	>100	>305
Derivative E	0.851	0.164	>100	>622
	0.300	0.152	11.4	74.6

it was clearly negative (Table 2). Tetracycline has been shown to induce phototoxicity [7], although relatively high patient doses are required. Doxycycline induces a clear dose-dependent increase in phototoxicity at serum levels of 2 μg/ml or greater [7]. Both tetracycline and doxycycline produced clear phototoxic responses in the assay. The IC50 values (+UV) were appreciably lower for doxycycline than for tetracycline (Table 1).

The assay is reproducible, particularly for determining the IC50 values for a given compound. Several CMTs were evaluated at different times under code. The results of these assays are given in Table 2.

The toxicity of the CMTs is dependent of the pH of the medium. Initial phototoxicity assay validation studies were conducted by using Earl's Balanced Salt Solution (EBSS) to dilute the test articles for application to the cells rather than HBSS. EBSS relies heavily on bicarbonate buffer and a CO_2 atmosphere to maintain the desired physiological pH. When the plates are removed from the 5% CO_2 atmosphere during UV (or dark) exposure, the pH of the EBSS climbs to 8. In contrast, HBSS is less dependent on CO_2/bicarbonate and maintains its pH better in room air. In an early assay conducted on the CMTs prepared in EBSS,

Table 2. Reproducibility of the assay: Comparison of multiple coded samples of select test articles.

TEST ARTICLE	MPE	IC50 (μg/ml)		PIF
		+ UV	–UV	
MINOCYCLINE	0.339	42.9	>100	>2.3
	0.121	58.2	>100	>1.7
	0	>100	>100	none
	0	>100	>100	none
Derivative B	0.333	12.0	65.8	5.5
	0.615	21.2	>100	>4.9
	0.178	>100	>100	none
	0.550	24.95	>100	>4
Derivative F	0.948	0.51	>100	>196
	0.991	0.45	>100	>222
	0.845	0.499	>100	>208
	0.691	0.326	>100	>305
Derivative E	0.937	0.092	>100	>1000
	0.921	0.098	>100	>1000
	0.851	0.164	>100	>622
	0.300	0.152	11.42	74.6

Table 3. Impact of choice of Balanced Salt Solution (pH) on the cytotoxicity of the CMTs.

	EBSS		HBSS	
Sample Number	IC50 (μg/ml)		IC50 (μg/ml)	
	(+UV)	(–UV)	(+UV)	(–UV)
1	4.21	>100	0.51	>100
2	>100	>100	>100	>100
3	47.3	>100	17.8	>100
4	0.57	>100	0.092	>100
5	17.4	>100	3.24	74.9
6	>100	>100	21.2	>100
7	>100	>100	58.2	>100

the IC50 values (+UV) were 2–10 fold higher (less toxic) than when the same CMTs were tested in HBSS (Table 3).

Chlorpromazine is the positive control tested with each assay and its response forms the basis for accepting or rejecting the assay trial. Depending on the material under test, either EBSS or HBSS may be used to prepare the chlorpromazine control. Table 4 summarises the historical data from both treatment buffers.

This is one of the first published reports in which the EU/COLIPA 3T3 phototoxicity assay protocol was used to evaluate a single class of chemicals. Tetracyclines, as a chemical family, have been reported to show phototoxic potential ranging from none to moderate, both *in vitro* and *in vivo* [6–9]. The current studies were performed as part of a larger, more extensive drug development programme. At present, the *in vivo* data on the phototoxic action of the CMTs is limited and proprietary. However, the data available correlate very closely with the phototoxicity rankings predicted by this *in vitro* assay. The *in vitro* assay allows for the assessment of phototoxic potential very early in the drug

Table 4. Summary of the data from the chlorpromazine assays.

Chlorpromazine prepared in:	Light exposure	IC50 μg/ml	Standard Deviation	Coefficient of Variation
EBSS	+UVA	0.60	0.24	40%
(n=9)	–UVA	9.57	1.24	13%
HBSS	+UVA	1.04	0.34	33%
(n=22)	–UVA	26.34	5.33	20%

development process, when only very small amounts of compound may be available.

The CMTs have potential applications for intervention in diseases associated with connective tissue breakdown, such as inflammation and cancer metastasis. Certain side-effects, such as phototoxicity, may interfere with specific clinical applications. What may be tolerated in the acute treatment of a life-threatening disease might be unacceptable when chronic treatment is required. These data can be used in the selection of lead compounds for specific applications. Those CMTs less phototoxic than minocycline would be candidates for all applications, including those requiring chronic exposure. The middle group might be acceptable for many applications, while those CMTs showing phototoxicity greater than or equal to doxycycline might need special efficacy to justify their use. Without modification to reduce their phototoxicity, they would have a more limited clinical application.

Conclusions

The 3T3 neutral red phototoxicity uptake test has been successfully used to evaluate a group of over 30 CMTs. Where human response data are available for the tetracyclines, the assay correctly ranked the intensity of the *in vivo* response. This assay has potential application in pharmaceutical development where UV, and particularly UVA, absorption is expected in the chemical class. The selection of the balanced salt solution, and the resulting pH of exposure, may have an impact on the solubility and therefore the toxicity of certain classes of chemicals.

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